

PROGRESS REPORT ABSTRACTS



MICROBIOLOGY BRANCH

Office of Naval Research



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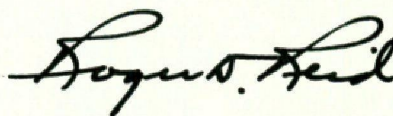
FORE WORD

This publication consists of brief reports on current research sponsored by the Microbiology Branch of the Office of Naval Research. It is presented in the interest of coordinating results of related investigations and of stimulating further exchange of scientific information. The present volume is offered in recognition of the enthusiastic response to previous issues.

The opportunity to review these preliminary and previously unreported results is to be respected. Those who read these abstracts should be aware that they contain material which is usually transmitted in a very personal way. With this in mind, you are asked to regard these reports as **PRIVILEGED PERSONAL COMMUNICATIONS**.

This printing does not constitute publication in the usual sense. It is anticipated that more complete reports will appear later in established scientific periodicals. Reference should not be made to these abstracts without permission, and then only as personal communications.

Credit for this volume belongs to the investigators who conducted the research and supplied the abstracts. ONR takes pride in the high quality of the investigations which it is our privilege to sponsor.



ROGER D. REID
Director
Biological Sciences Division

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ABSTRACTS

STUDIES ON PASTEURELLA PESTIS

D. M. Eisler and E. K. von Metz

Naval Biological Laboratory, University of California

ASSISTED BY

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

To increase knowledge of Pasteurella pestis infections by studies on (a) rapid modification of infections in mice by Fraction IB (FIB) of P. pestis, (b) modification of the hemagglutinin test to use protein antigen to detect antibodies of P. pestis in mouse sera, and (c) the significance of "normal" precipitins in animal sera against bacterial antigens as detected by the agar diffusion technique.

ABSTRACT

(a) FIB, a lipoprotein extracted from P. pestis, has been shown to increase resistance of mice to infection with P. pestis and to enhance susceptibility to infection with Pseudomonas pseudomallei when the material was subcutaneously injected concomitantly with virulent organisms. The effective action appears to take place within 72 hours of inoculation. Bacteriological, histological and immunological observations indicate that neither early specific antibody formation nor phagocytosis is responsible for early interference of infection with P. pestis. Present studies are designed to determine the mechanism involved.

(b) Hemagglutinin titers with FIB mouse antisera and normal or tanned mouse red blood cells (rbc) treated with FIB are lower than those with tanned sheep rbc treated with FIA/B, a carbohydrate-lipoprotein. Attempts are being made to improve the hemagglutination reactions with FIB and homologous mouse antisera for studying the immunological aspects of the protective effect described above in (a).

(c) The Ouchterlony agar diffusion technique revealed precipitation reactions between FIB rabbit and mouse antisera and Proteus mirabilis antisera and the following antigens: FIB of P. pestis, suspensions of P. pestis, strain EV76, Shigella flexneri, serotype B-3, Escherichia coli, a lactose nonfermenting Gram-negative bacillus and P. mirabilis. Normal sera of rabbits, mice, man, cow, sheep, swine and guinea pig also reacted in a limited way with these antigens. The presence of these "normal" precipitins may be of significance in the interpretation of the specific antigen-antibody reactions.

PLANS FOR FUTURE

(a) To continue studies on the biological activity of FIB in modifying infection in mice by P. pestis, and the mechanism by which the lipoprotein mediates its protective action in the host, (b) to improve the optimal conditions for use of hemagglutination tests for detection of FIB antibodies to P. pestis, (c) to determine the relation of coagulase production by P. pestis to strain virulence, (d) to determine the

effect of pentoses on morphology and virulence of *P. pestis* under conditions of constant growth.

CURRENT REPORTS AND PUBLICATIONS

(a) D. M. Eisler and E. K. von Metz, "Antibodies in the sera of normal animals and those immunized with Fraction IB of Pasteurella pestis" In preparation for publication.

THE BIOLOGICAL EFFECTS OF LIGHT (SMALL) AIR IONS

A. P. Krueger and R. F. Smith

Naval Biological Laboratory, University of California

ASSISTED BY Capt. C. E. Meyers, Dr. G. J. Hildebrand, Cdr. J. W. Millar

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

To determine the biological effects of light air ions. Specifically, (a) to measure the parameters of gaseous ion effects on the mammalian trachea, (b) to observe the effects of air ions on the trachea of primates, (c) to investigate the action of air ions on certain enzyme systems, (d) to determine which gaseous ions affect tracheal functions other than ciliary activity.

ABSTRACT

Groups of mice exposed to high densities of unipolar light air ions for 72 hours exhibited persistent alterations in the functional efficiency of their tracheas. These effects lasted at least 4 weeks, and in the case of animals treated with (+) ions, included diminished ciliary activity, pale contracted tracheal mucosa, and enhanced vulnerability to trauma.

Following treatment with (-) ions, animals displayed increased ciliary activity with no other detectable changes. It required at least 60 minutes of exposure to ions to induce such "permanent" functional changes.

The minimal ion densities producing changes in ciliary activity within an arbitrary period of 30 minutes were determined with extirpated tracheal strips from rabbits and guinea pigs. The threshold value for (-) ions was approximately 2.5×10^3 ions/cm²/sec. and that for (+) ions was in the range between 1×10^4 and 2.5×10^5 ions/cm²/sec.

The evidence indicates that ion-induced functional changes in the ciliated epithelium of the pulmonary tree are the results of direct contact of ions with surface cells and do not involve participation of the central nervous system or circulation. So far as ciliary activity is concerned, the number of ions required to produce a change in rate is very small.

As is true with mice, rats, guinea pigs and rabbits, the exposure of monkeys to negative air ions enhanced the efficiency of the tracheal surface clearing mechanism, while positive ions depressed it. In tissues untreated with air ions the minor trauma produced by touching the surface with a probe induced no more than a brief vascular reaction, but after exposure to positive ions, this procedure resulted in prompt production of a well-defined ecchymotic area.

Tracheal strips from two humans were removed at autopsy, and the effects of unipolar ions on ciliary activity were observed. Negative ions increased the rate of ciliary beat, and positive ions decreased it.

We have shown that negatively ionized oxygen is the actual physiolog-

ical mediator of negatively ionized air. Recently we found that such negative ions can increase the rate of conversion of succinate to fumarate in a modified Keilin-Hartree pig heart homogenate. Further localizing experiments indicated that negative ions accelerate the re-oxidation of reduced cytochrome c by acting on cytochrome oxidase. These results suggest that many of the physiological effects of negative air ions are due to the action of negatively ionized oxygen on certain iron porphyrin compounds.

By providing rabbits with a separate airway for respiration and using a tracheal aperture cephalad to the point of intubation for observation, it was possible to expose the living trachea to gases such as N_2 and CO_2 . We found that positively charged CO_2 is the agent responsible for all observed functional changes including ciliary activity, smooth muscle contracture, mucosal ischemia, and enhanced vulnerability to trauma.

PLANS FOR FUTURE

Work on (a) the mechanism of action of positively ionized CO_2 , (b) the effects of ionized air on the hypersensitive state, (c) the effects of ionized air on susceptibility to airborne infection, (d) collaborative experiments designed to determine the effects of air ions on macrophage activity in tuberculosis and the clearing of airborne nonpathogens from the human respiratory tree.

CURRENT REPORTS AND PUBLICATIONS

(a) A. P. Krueger and R. F. Smith (1959), "Parameters of gaseous ion effects on the mammalian trachea." J. Gen. Physiol. 42, 959-969

(b) A. P. Krueger, R. F. Smith and J. W. Millar (1959), "Effects of air ions on the trachea of primates." Proc. Soc. Exptl. Biol. Med., 101, 506-507

(c) A. P. Krueger and R. F. Smith (1959), "An enzymatic basis for the acceleration of ciliary activity by negative air ions." Nature, 183, 1332-1333

(d) A. P. Krueger and R. F. Smith (1959), "The physiological significance of positive and negative ionization of the atmosphere." J. Roy. Soc. Health, 79, 642-648

(e) A. P. Krueger and R. F. Smith, "Further studies of gaseous ion action on the trachea." Proc. Soc. Exptl. Biol. Med. In press.

MELIOIDOSIS: IMMUNIZATION AND PATHOGENESIS

Russell Miller, Jr., and Robert J. Heckly
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ASSISTED BY Doris Clinger, Melvin Klumpp and T. R. Elliott

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

(a) Immunization against melioidosis, (b) studies on the pathogenesis of melioidosis, (c) purification and characterization of toxic substances in culture filtrates of Pseudomonas pseudomallei which have lethal, enzymatic and necrotizing properties.

ABSTRACT

(a) Immunization. During the past year experiments directed toward the development of a vaccine for immunization against melioidosis have been continued. Inasmuch as the final preparation is to be used in man, study has been limited to the use of vaccines prepared from killed P. pseudomallei, strain 114-8, and a mucoid variant of this strain, or soluble products therefrom. It has not been possible to repeat the earlier results (immune index of 700) previously reported (CNR Report ACR 35). The suspicion that formaldehyde denaturation of protein decreased the antigenic integrity of vaccine preparations has led to the use of ethanol and ethylene oxide as killing agents. Such preparations have proved equally unsatisfactory. Neither has it been possible to enhance the protective value of vaccines by increasing the antigenic stimulus to the maximal tolerated doses, not by prolongation of the period of immunization. The addition of excipients such as alum, mineral oil or Freund's complete adjuvant has not been helpful. In general, mice can be protected against approximately 100 LD₅₀ of virulent organisms when challenged by the intraperitoneal route; it has not been possible to protect them against inhalatory challenge.

Sera of rabbits and primates have been analyzed electrophoretically during the course of immunization. A significant increase in alpha globulins was noted concomitant with minor decreases in both beta and gamma globulins.

(b) Pathogenesis. Two specialized surgical techniques have been developed to study the pathogenesis of melioidosis. It is now possible to observe the developing lesions of the disease in the rabbit ear chamber and in the viscera (some surgically transposed) of rabbits provided with a plastic abdominal window. Because rabbits with melioidosis develop infectious ocular and nasal discharges, it has been necessary to design and construct specialized animal holding units to permit the taking of both still and cine photographs (both macro- and micro-) with maximal safety. In contradistinction to recorded pathology in man, resolving lesions had not heretofore been observed by us in infected animals.

However, it has now been possible to document lesions in the rabbit ear chamber that could only be classified as subacute on the basis of morbid histopathology.

(c) Purification and characterization of toxic substances in culture filtrates. Studies on toxic culture filtrates have been continued with emphasis on the characterization of the proteolytic enzyme rather than on purification. The enzyme was stable between pH 6 and 9, and its maximal activity rate for casein digestion was near pH 7.5. It was inactivated by heating to 60 C for 30 min, but not by ethanol or acetone at room temperature or by repeated freezing and thawing. The electrophoretic mobility was about -0.15×10^{-5} cm²/sec/volt, and the sedimentation coefficient was about 3.6×10^{-13} cm/sec/dyne/g.

PLANS FOR FUTURE

(a) To continue study of methods of immunization against melioidosis using killed P. pseudomallei, (b) to fractionate immune sera electrophoretically and determine if agglutinins, precipitins and complement-fixing antibodies are associated with particular fractions, (c) to compare and document photographically the lesions of melioidosis in infected control animals, immunized animals and infected animals receiving chemotherapy, (d) to continue studies on the purification of the biologically active components in culture filtrates in order to characterize them more completely, both physically and chemically.

STUDIES OF PROBLEMS OF SAFETY IN THE
MEDICAL MICROBIOLOGY LABORATORY

C. Lamanna, M. A. Chatigny, and R. J. Heckly
Naval Biological Laboratory, University of California

ASSISTED BY

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

To determine the nature of the dangers to which workers in medical microbiology laboratories are exposed, and to develop effective practical means for reducing these dangers.

ABSTRACT

Commercially available pliable, autoclavable and heat-sealable plastic sheeting has been found to be economical and practical to use to make safe enclosures for isolating the laboratory worker from bench type laboratory equipment harboring infectious material.

In reviewing the possibilities existing at the Naval Biological Laboratory for the inadvertent exposure of the laboratory worker to infectious material, the open front safety bacteriological hood has been found to be deficient.

PLANS FOR FUTURE

To continue the review of hazards existing in the laboratory, and to work on solutions of problems as the problems are discovered. To produce a motion picture illustrating some of the safety problems encountered, and procedures and equipment developed at NBL to solve these problems.

CURRENT REPORTS AND PUBLICATIONS

C. Lamanna (1960), "Use of autoclavable plastic material for do-it-yourself solutions to laboratory problems of safety." J. Lab. Clin. Med. In press.

STUDIES ON THE EFFECTIVE TOXIC PARTICLE
SIZE OF BOTULINUM TOXIN

R. J. Heckly, G. J. Hildebrand and C. Lamanna
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ASSISTED BY J. Ng, M. N. Klumpp and T. R. Elliott

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

(a) To determine the particle size of the toxin which passes into the lymph and hence into the blood stream from the intestines of animals which have ingested botulinum toxin, (b) to determine as precisely as possible the probable limits of the particle size or sizes of the toxic unit which reaches the effector sites.

ABSTRACT

Lymph, collected from rats which had been administered intraduodenally large amounts (up to 15×10^6 mouse LD_{50}) of crude or crystalline botulinum toxin, was collected by cannulation of the cisterna chyli. The sedimentation coefficient, and hence the particle size of the toxic unit in the lymph, was determined using partition cells and sedimentation in density gradient tubes. It was found that the particle size of the bulk of the toxin in the lymph was considerably less than that of the crystalline toxin which was fed. Exact values have not yet been determined, but the sedimentation coefficient appears to be less than 10×10^{-13} cm/sec/dyne/g, and possibly as low as that of serum albumin.

PLANS FOR FUTURE

Studies on the particle size of toxin passing through capillary walls and other biological membranes is being continued. With refinements of the techniques, it is hoped that the molecular weight of the particles which could reach the effector sites can be estimated.

CURRENT REPORTS AND PUBLICATIONS

None

MELIOIDOSIS: DIAGNOSTIC PROCEDURES

Clara Nigg

Naval Biological Laboratory, University of California

ASSISTED BY Margaret Johnston

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

To develop reliable serological tests for the diagnosis of melioidosis.

ABSTRACT

An improved complement-fixing (CF) antigen with a titer of 1-8000 has been developed since the preceding report was submitted. This antigen is an aqueous extract of Pseudomonas pseudomallei, disrupted by sonic treatment.

Having no sera from human cases of melioidosis at our disposal for evaluating our CF test, the development of CF antibodies has been studied in experimentally infected laboratory animals. None of 15 infected rabbits developed positive CF reactions within 3 to 5 days postinfection, but over 50 per cent (5 of 8 rabbits) were positive within 7 to 8 days, and 100 per cent (5 of 5 rabbits) were positive at 9 to 11 days. This latter interval would probably coincide with onset of symptoms in human infections. If so, such an early diagnosis should make it possible to institute appropriate and adequate early therapy. The titers in infected rabbits reached 1-640 providing therapy, which was necessary for the survival of the rabbits during the observation period, was maintained at minimal levels. Similar results were obtained in infected guinea pigs.

The results in monkeys (one infected intraperitoneally and one with an aerosol) were of special significance since an infecting dose of ca 1/100 LD₅₀, which was inadequate to produce any clinical signs of infection, nevertheless stimulated the production of CF antibodies with titers of 1-20 and 1-40 within 18 and 16 days respectively, postinfection.

The production of demonstrable CF antibodies after such a small infecting dose was interpreted as indicating that the organisms must have multiplied (inducing a subclinical infection) to produce sufficient antigen to account for the CF titers obtained. The titer of one monkey returned to the base line within 11 wk postinfection, at which time a second infecting dose (ca 1 LD₅₀) resulted within 8 days in a very sharp rise in titer to 1-640. If such an anamnestic reaction can be produced with the inoculation of one dose of killed vaccine, this would indicate a method which might be applied to population surveys for uncovering evidence of subclinical infection in man in those areas where the disease occurs naturally. This, in turn, would throw some light on epidemiolog-

ical aspects of melioidosis.

Normal sera from man and animals have been tested to determine the specificity of the test. CF reactions were negative in 1-5 dilution of the following normal sera tested to date: 30 sheep, 13 goats, 40 rabbits and 71 human sera. A few weak nonspecific reactions in lower dilutions were encountered among these normal sera.

PLANS FOR FUTURE

(a) To study the anamnestic effect of killed vaccines on the CF titer of subclinically infected monkeys (b) to examine the possibility of (1) applying the CF diagnostic test to cases of human melioidosis and (2) undertaking investigations bearing on the existence and prevalence of subclinical infection in endemic areas (c) Development of hemagglutination test for the diagnosis of melioidosis.

CURRENT REPORTS AND PUBLICATIONS

Nigg, C. "The serological diagnosis of melioidosis." In preparation.

MELIOIDOSIS: CHEMOTHERAPY

Clara Nigg and Margaret Johnston
Naval Biological Laboratory, University of California

ASSISTED BY

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

Chemotherapy in experimental melioidosis.

ABSTRACT

The efficacy of several therapeutic agents in mice infected intraperitoneally (ip) with a virulent strain of Pseudomonas pseudomallei was presented in the preceding report. It was also stated that the therapeutic efficacy of the same agents was significantly lower in mice infected with aerosols. It has, however, been assumed in this laboratory that this difference was more apparent than real and was referable to the fact that therapy was instituted within one hour after ip infection but delayed 24 hr in inhalatory infections for safety purposes. Because of the delay in treating animals infected with aerosols, we have assumed that the latter procedure (which was routine) constituted a more rigid test of therapeutic efficacy. In tests designed to clarify this point, it was found that the curative effect of Sulfose was comparable in mice infected either with aerosols or by the ip route, if therapy in both groups was instituted immediately after infection. Thus, melioidosis can be successfully treated regardless of the route of infection, the efficacy depending on the promptness in instituting therapy.

During the past year, emphasis has been placed on combined therapy in inhalatory infections in which therapy was delayed 24 hr postinfection. Additional assays of Sulfose plus dihydrostreptomycin in mice infected with 2857 LD50 resulted in 80 per cent cure, confirming the high rate of cure reported previously. With the dosages used in these assays, Sulfose alone cured only 20 per cent, and dihydrostreptomycin none.

Although preliminary results with Aureomycin plus Albamycin were reported to be as promising as those of the above combination, subsequent assays resulted in 70 and 50 per cent cures in mice infected with 233 and 1333 LD50, respectively, indicating that this latter combination was probably somewhat less effective. With the dosages used in these assays, Albamycin alone effected no cures, while Aureomycin cured only 20 per cent in one experiment and none in the second.

No additional assays have been made to date with Signemycin V which had shown some promise.

The therapeutic results in preliminary assays of Panalba (tetra-

cycline and novobiocin), Madribon (long-acting sulfadimethoxine), and Kantrex (kanamycin sulfate) were not sufficiently encouraging to warrant further study.

PLANS FOR FUTURE

Continued study of combined therapy in experimental melioidosis with emphasis on inhalatory infections.

INVESTIGATION OF BIOPHYSICAL TECHNIQUES

Robert J. Heckly and Nelson Newton

Naval Biological Laboratory, University of California

ASSISTED BY Melvin Klumpp and T. R. Elliott

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

(a) To develop facilities and techniques so that various physical and chemical measurements can be made with extremely small amounts of purified biological materials or on impure preparations, (b) to develop and apply specific methods for the purification of viruses, toxins, enzymes and other biological materials.

ABSTRACT

A method has been developed to obtain accurate measurements of electrophoretic mobilities of biologically active substances at extremely low concentrations even in the presence of impurities. After conventional free boundary electrophoretic separation, the contents of the cell were fractionated and these fractions were then assayed by appropriate tests to obtain the data for calculating mobilities.

A method has been developed for the purification of vesicular stomatitis, vesicular exanthema and infectious bovine rhinotracheitis virus employing zinc hydroxide, differential centrifugation, and finally centrifugation on a density gradient column. Electron microscopy of purified virus indicated that vesicular exanthema virus has a particle size of 55 μ , a sedimentation coefficient of order of 200×10^{-13} cm/sec/syne/g. Spectrophotometric analysis shows a pronounced peak at 260 μ and a protein to nucleic acid ratio of 1.69. Such a ratio is consistent with a high degree of purity for a nucleoprotein.

A system for continuous counter-current dialysis has been developed to facilitate the processing of large volumes of solutions with a minimum of reagent. The method has been applied successfully to the precipitation of toxin, at a final concentration of at least 80 per cent ethanol, from culture filtrates of Pseudomonas pseudomallei. In a typical experiment 10 liters of 95 per cent ethanol were required for every 14 liters of filtrate.

Facilities have been developed to determine the sedimentation coefficient, and hence particle size, of viruses and toxins in the presence of large amounts of impurities. This has been essentially the application of conventional partition cells and density gradient ultracentrifugation techniques which have been developed in the last few years.

Progress has been made in developing a suitable method for determining the partial specific volume of virus or other material using small amounts of the substance. The method showing the greatest promise, because of its extreme accuracy, is a magnetic float method for measuring the change in density of a solution as a function of the solute concentra-

tion.

PLANS FOR FUTURE

Studies are being continued on methods for separating and purifying biological materials and on methods for making various physical or chemical measurements on these substances.

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(b) N. Newton and R. E. Bevis (1959), "Purification of animal viruses with $Zn(OH)_2$." Virology, 8, 344-351

(c) R. J. Heckly (1959), "An apparatus for continuous counter-current dialysis." Biochem. et Biophys. Acta, 35, 548-549

RAPID METHODS FOR THE DETECTION AND IDENTIFICATION
OF **SELECTED** PATHOGENS

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TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

(a) Methods for isolation of pathogenic microorganisms, (b) methods for accelerating growth of pathogens, (c) methods for rapid identification of microorganisms and their products.

ABSTRACT

Oral flora were inhibited in growth on a general purpose medium (SB) by penethamite HI (I) or Na hydrosulfite (II). Growth of Pasteurella tularensis, Pseudomonas pseudomallei and Coccidioides immitis was not inhibited. II, but not I, was toxic to Pasteurella pestis. Gentian violet, in addition to I, further inhibited oral yeasts, with no effect on these organisms.

D-histidine in SB medium was shown to be inhibitory to Brucella suis but not to P. tularensis. Phosphate in this medium inhibited P. tularensis only. Other compounds were assessed during a comparative study of the nutrition of these species growing on solid media.

Studies have revealed lysogenic strains of P. pseudomallei, some of which can be induced by ultra violet light (UV). One strain of P. mallei, when lysogenized by a P. pseudomallei phage, can also be induced by UV. Lysogenic strains of oral streptococci and staphylococci were not unequivocally induced by UV.

Host range and specificity were determined for samples of diagnostic Brucella bacteriophage of Russian origin. No non-Brucella species were lysed; likewise, not all strains of the 3 Brucella species tested were lysed.

For work on the use of fluorescent antibody in rapid serological identification of microorganisms, the Scopicon microprojector light source and optics were shown to be adaptable and adequate as a UV illuminating device. This extends the usefulness of this technique to installations presently equipped with this microprojector.

Work is in progress on the serological differentiation of Bacillus anthracis from Bacillus cereus, using spore and vegetative cell antigens and antisera.

A literature survey was completed on compounds restricted in distribution to microorganisms. The occurrence, properties and assay of diamino-pimelic acid, dipicolinic acid, muramic acid, B-hydroxybutyric acid, D-aminoacids and chitin were discussed. The applicability of these compounds to a method for rapid identification was considered.

A literature survey was completed on the general subject of

decontamination at low temperatures. Factors affecting survival of microorganisms, biological activity and disinfectant activity were discussed along with a consideration of problems which might be encountered in an arctic-type environment. Methods presently available for sanitation and decontamination were also discussed.

Fifty-nine isolates from specimens of snow, ice, earth, etc., collected in the Antarctic are being tested with regard to growth response to temperatures ranging from 1 C to 37.5 C. Some cultures have been found to grow at 1 C - 2 C, but not at 22 C - 25 C. Results are too early for assay, but it appears that some specimens contained bacteria which gave similar plate counts at 1 C - 2 C as at higher temperatures.

Studies on the antigenic and immunogenic properties of *Shigella* are being conducted. Strains grown in vivo and in vitro in several systems are being compared in an attempt to correlate antigens present with immunogenic and pathogenic activity and with precipitation patterns demonstrable by gel-diffusion.

PLANS FOR FUTURE

(a) Convert the improved "all-purpose" medium to various media, each of which will be selective for a narrow group of organisms, (b) continue study of the Russian isolated Brucella bacteriophage, with attempts to increase its intra-genus host range, (c) investigate the feasibility of using the fluorescent antibody technique for detection of specific groups of microorganisms recovered directly from the aerosol state, (d) investigate the feasibility of mechanizing the steps in the fluorescent antibody technic for identification of microorganisms (with M. A. Chatigny), (e) continue studies on the serological differentiation of B. anthracis from B. cereus.

CURRENT REPORTS AND PUBLICATIONS

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(c) W. D. Won and O. G. Lien, Jr. (1958), "Fish hydrolyzate media for Pasteurella pestis." *Am. J. Hyg.*, 66, 81-84

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(g) H. Wolochow, "Properties of a bacteriophage for Malleomyces pseudomallei." In preparation.

(h) H. Wolochow, "Studies on the nutrition of Brucella suis and Pasteurella tularensis." In preparation.

(i) C. Lamanna and D. Eisler (1960), "Comparative study of the agglutinin of the endospores of Bacillus anthracis and Bacillus cereus." *J. Bacteriol.* In press.

STUDIES OF MAN AS A DETECTOR OF MICROORGANISMS IN AEROSOLS

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ASSISTED BY J. P. Hresko, T. Reid, P. Wagner, R. F. Smith

TASK NO. NR 109-004

CONTRACT N7onr-29540 and
N7onr-29536

OBJECTIVES

A study of (a) physical, (b) physiological, (c) microbiological, factors influencing recovery of organisms from human oropharynx following exposure to microbial aerosols.

ABSTRACT

In continuation of studies of the recoverability of bacteria from the oropharyngeal region of humans exposed to aerosols of Serratia marcescens, a new series of exposures using the Wells atomizer is almost completed. This series is being done in order to classify subjects on the basis of sampling ability after exposure to bacterial aerosols, and is preliminary to exposure to viral aerosols.

Concurrent with routine sampling (culturing of gargle samples), samples were taken of nasal mucus. Per cent recovery from this latter material was substantially higher than from routine gargle specimens.

Methods of assaying bacteriophage from gargle samples are being studied. A method for assay of phage upon MF using the entire gargle sample is being studied.

The equipment necessary for the physiological studies of the subjects is being tested. The studies will include tidal volume, total volume, respiratory rate, functional residual volume, dead space, nasal vs mouth breathing, and influence of swallowing, exercise and posture upon individual sampling ability.

Work on the influence of air ions upon tracheal ciliary activity, and other physiological activities (in collaboration with Dr. A. P. Krueger) in animals, is being studied, and an apparatus for the exposure of subjects to air ions prior to exposure to bacterial aerosols is being constructed.

The passage of introduced bacteria through the intestinal wall is being studied, together with the role of the lymphatic system in dissemination of the organisms throughout the body. Surgical and technical methods have been developed which permit both the lymph and blood to be continuously sampled for five days following the surgical procedures. Bacteria have been cultured from the lymph after instillation (via polyethylene tube) into the intestine and the stomach, and from the lymph of animals recovered from surgery who were given the organisms in drinking water. The major area of intestinal passage of bacteria has been determined.

Following administration of the organisms via aerosol exposure,

Attempts to recover the bacteria from the left thoracic duct have been unsuccessful. Since lymphatic drainage from the lungs mainly takes place through the right thoracic duct, this source of lymph will be tested.

PLANS FOR FUTURE

(a) Collect physiological respiratory data on relevant parameters affecting recovery of microorganisms from the human oropharynx following exposure to aerosols. Parameters involved include: tidal volume, total volume, rate, functional residual volume, dead space. (b) Study correlation between humans on the basis of frequency of swallowing, consistency of saliva, drug effects. (c) Compare recovery of bacteria to that of bacteriophage and other innocuous viruses using selected subjects with predetermined recovery classifications. (d) Explore new methods for producing homogeneous aerosols of known particle sizes. (e) Following exposure to air ions, determine if the subject's recovery pattern changes. (f) Continue investigation of intestinal permeability and lymphatic distribution of bacteria.

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USE OF VARIOUS TISSUE CULTURE CELL LINES FOR THE ISOLATION
AND CHARACTERIZATION OF MAMMALIAN VIRUSES

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ASSISTED BY Norman B. Darby, Jr.

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

(a) To produce and characterize cell lines from a variety of domestic animals, (b) to develop methods for the long-term preservation of such cell lines, (c) to study the host range of various human and animal viral agents on these cell lines, (d) to characterize the various cells by their viral susceptibility, idiograms, potential malignancy, and antigenicity.

ABSTRACT

Four cell lines, the MDBK (bovine kidney), MDOK (ovine kidney), MDCK (canine kidney), and MDGK (caprine kidney), have been isolated and established. These cells have been shown to have a fairly wide host range for a variety of human and animal viruses, particularly the MDCK cell line. It is possible to store all cell lines by freezing for a period of at least one year.

PLANS FOR FUTURE

Studies have been initiated on the ability of the various animal cell lines to support the growth of adeno-viruses of man and will be extended to include certain of the Coxsackie group. Attempts to isolate cell strains of equine and feline origin will be continued.

CURRENT REPORTS AND PUBLICATIONS

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(b) S. H. Madin and N. B. Darby, Jr. (1958), "Established kidney cell lines of normal adult bovine and ovine origin." Proc. Soc. Exptl. Biol. Med., 98, 574-576

(c) R. A. Crandell and S. H. Madin (1959), "Experimental studies on a new feline virus." Am. J. Vet. Research. In press.

PATHOGENESIS OF MAMMALIAN VIRUSES

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TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

To analyze the disease processes on a multicellular level produced by an epitheliotropic virus (vesicular exanthema of swine virus, VESV), a pantropic virus (swine X), and one which produces primary hepatitis (Friend murine hepatitis agent).

ABSTRACT

Vesicular exanthema of swine virus appears in both the blood and feces of animals between 24 and 96 hours after intradermal inoculation. The appearance of virus is correlated with the febrile and, to a lesser degree, with the appearance of lesions. The virus appears to multiply in the tongue and tonsils, in addition to the epidermis. Studies on swine X virus, while not yet as detailed as those noted above, indicate that the lesions and symptoms of the disease are due to multiplication of the virus in the endothelial lining cells of the small blood vessels, as well as the lymph nodes. Studies have just been initiated on the Friend hepatitis virus, and no significant data, as yet, is available.

PLANS FOR FUTURE

It is proposed to continue the studies discussed above and to extend them wherever possible. It is also proposed to attempt the propagation of swine X virus. Emphasis will be placed on attempting to propagate the Friend hepatitis virus in tissue culture.

GENETICS OF MAMMALIAN VIRUSES

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ASSISTED BY Adeline J. Hackett

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

(a) To describe and analyze the genetic behavior of selected mammalian viruses, (b) to find virus-cell systems in which genetic studies of human and animal viruses can be studied more suitably than at present.

ABSTRACT

Suspensions of vesicular exanthema of swine virus (VESV) contain two types of particles; one which is virulent in swine and produces a large, clear plaque in tissue culture cells, and a second which is avirulent in swine and produces a minute, opaque plaque on tissue culture cells. Only the large plaque type has been recovered from experimentally infected animals. However, marked variability of plaque type has been observed in tissue culture. Analysis of progeny from isolated minute plaques, and from single cells infected with minute plaque type, have shown the virulent plaque type to be present at a frequency of 0.1 per cent. The genetic basis of this variability in plaque morphology and, presumably, of virulence, is under investigation. Experiments are in progress to include the role of inactive virus or of multiple infections of cells with mixed plaque types as a source of the observed variability.

PLANS FOR FUTURE

A solution to the problem of plaque type variation is required before a systematic genetic investigation of VESV can be undertaken. An examination of a large number of independently occurring minute variants for more stable strains is contemplated, as well as a search for other characteristics, such as host range differences, which would be suitable genetic markers for recombination studies.

IN VITRO BIOLOGY OF CYTOCIDAL MAMMALIAN VIRUSES

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ASSISTED BY Adeline J. Hackett and Robert E. Bevis

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

(a) To characterize the behavior of selected mammalian viruses as it pertains to the general problem of viral multiplication, (b) to accumulate information which may pertain to a concurrent genetic study of these viruses.

ABSTRACT

Two groups of viruses are currently under investigation; those of vesicular stomatitis (VSV) and of vesicular exanthema of swine (VESV). Primary attention with VSV is centered on a transmissible interfering component (T) produced during virus multiplication in chick embryo tissue cultures. This component, which possesses many of the characteristics of influenza virus "interferon", is recognized by its capacity to suppress the multiplication of the infective particle. Experiments are being carried out to separate T from virus particles in order to describe its chemical and physical nature, the conditions of its formation, immunological relationships, and its role in the multiplication of related and unrelated viruses.

An intensive study of two plaque variants of VESV, E₅₄ type, is being made, correlating virus multiplication and cytopathology in a clonal derivative of swine kidney cells. Cytopathology is being examined in H and E preparations, by Feulgen, acridine orange and fluorescent antibody techniques, and by electron microscopy.

PLANS FOR FUTURE

A continuation and intensification is planned of the studies described above.

CURRENT REPORTS AND PUBLICATIONS

Mary E. McClain and Adeline J. Hackett (1959), "Biological characteristics of two plaque types of VESV, E₅₄." Virology. In press.

STUDIES ON THE SURVIVAL OF AIRBORNE MICROORGANISMS

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ASSISTED BY E. Boerke, J. Due, S. Dunn, D. Flemming, W. Shupe, R. Thompson

TASK NO. NR 109-004

CONTRACT N7onr-29536

OBJECTIVES

(a) To study the various intrinsic biological factors and environmental circumstances which affect the survival and infectivity of airborne microorganisms, (b) to collect data which might ultimately lead to an understanding of the mechanisms involved in the death of airborne microorganisms.

ABSTRACT

Utilizing stirred settling chambers, the effects of relative humidity and temperature on airborne Serratia marcescens have been investigated. This organism exhibits a minimal airborne survival within a rather narrow range of relative humidity around 48 per cent at 25 C. The effects of such factors as suspending fluid and of culture age upon aerosol behavior as a function of relative humidity are being studied.

Data are being collected on the airborne stability of Pasteurella tularensis maintained at various relative humidities and temperatures in the rotating drums which were developed in this laboratory. This organism appears to survive optimally as an aerosol at relative humidities above 80 per cent at 27 C. Of numerous strains of P. tularensis which have been tested for aerosol survival capacity, none has exhibited a significantly different behavior.

In an investigation on the effects of ultraviolet radiation on airborne microorganisms, a series of experiments has indicated that opaque substances, such as nigrosine or carbon black, have a capacity to protect airborne microorganisms against the lethal effects of solar radiation.

A preliminary study has indicated that Yellow Fever Virus in the airborne state is best able to survive in the low ranges of relative humidity (less than 30 per cent at 27 C).

PLANS FOR FUTURE

(a) To study the effect of cell age, as established in synchronous cultures, on the airborne survival of microorganisms, (b) to relate the "indicated" aerosol concentration, as determined by animal exposure, in aged aerosols of Pasteurella tularensis, (c) to study the effects of solar radiation on bacterial and viral aerosols.

CURRENT REPORTS AND PUBLICATIONS

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RESEARCH ON THE MODE OF ACTION OF
ANTIBIOTICS ON BACTERIA

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ASSISTED BY Marj Bohnhoff

TASK NO. NR 103-013

CONTRACT Nonr-2701(00)

OBJECTIVES

The objective of this investigation is an explanation of the enhanced susceptibility of mice to infection with Salmonella enteritidis (inoculated by mouth) following treatment the day before with a single large dose of streptomycin (inoculated by mouth).

ABSTRACT

CF-1 mice used in this investigation required inoculation of $10^5 - 10^6$ microorganisms to infect 50% with the test strain of S. enteritidis. The day after a single dose of 30-50 mg of streptomycin by mouth <10 Salmonella sufficed to infect half the mice.

Inoculations of suspensions of feces from normal mice into streptomycin treated mice significantly reduced their susceptibility to Salmonella infection. Anaerobic cultures of feces were also effective, but anerobic cultures were not.

It has been shown that the bowel content of normal mice, particularly the upper half of the colon, contains a substance which inhibits multiplication of Salmonella in vitro. This bacteriostatic activity is demonstrable in supernates of bowel content after filtration or heating to 100 C.

After administration of streptomycin, the bowel content is no longer inhibitory. Activity is restored after administration of suspensions of colon content from normal, untreated mice.

An inhibitory substance with similar properties is elaborated by several strains of Bacteroides during prolonged cultivation in thioglycollate media. Supernates of such cultures are bactericidal for Salmonella at pH 6.0 and bacteriostatic at somewhat higher pH. The inhibitory substance is an acid, heat stable, readily dializable and ether soluble.

Germ free mice obtained from the Lobund Institute of the University of Notre Dame were as susceptible to inoculation of Salmonella by mouth as streptomycin-treated CF-1 mice. However, establishment of Bacteroides in the gut of germ free mice failed to prevent infection with Salmonella.

PLANS FOR FUTURE

Continue in vivo and in vitro studies to determine the nature of the resistance of normal mice to Salmonella infection.

PHYSIOLOGICAL ACTIVITIES OF MARINE BACTERIA
WITH SPECIAL REFERENCE TO INCREASED PRESSURE

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ASSISTED BY Leslie Ralph Berger and Fred E. Palmer

TASK NO. NR 103-020

CONTRACT N6onr-27518

OBJECTIVES

(a) To study the occurrence, characteristics, and physiological activities of bacteria with particular reference to their effects on biological, geological, and chemical conditions in the sea, and (b) to obtain quantitative information on the ways in which bacteria are affected by marine environmental conditions, especially high pressures such as occur in the deep sea.

ABSTRACT

The deep sea is characterized by temperatures in the neighborhood of 3°C and by hydrostatic pressures which increase with water depth by approximately 0.1 atm per meter. Such low temperatures and high pressures retard the physiological activities of most terrestrial bacteria, and have diverse effects on deep-sea forms. Although pressure per se affects the volumes of large molecules like proteins and the reaction rates between molecular species, these effects are influenced markedly by the temperature and chemical composition of the medium. While the investigations have been aimed at explaining conditions of life in the deep sea, attempts are being made to explain the fundamental mechanisms that are involved.

In attempting to account for the basic metabolic differences between bacteria which tolerate or require high hydrostatic pressures and those which cannot live under such conditions, Dr. Berger has examined the in vitro phosphatase activity of three separate systems. The source of two of the three enzyme systems were bacteria, Bacillus abyssus and B. borborokoites, selected from screening a large number of organisms. These two bacteria, originally isolated from appreciable oceanic depths, were chosen, because simple extracts gave preparations of alkaline phosphatases with relatively high specific activities. The third phosphate employed was a commercially purified product derived from calf intestine.

Enzymatic activity was measured by determining the rate at which p-nitrophenylphosphate was hydrolyzed. The p-nitrophenol, resulting from the hydrolysis of the substrate, was determined colorimetrically. All experiments were made at the pH optimum for each enzyme and at an enzyme concentration which gave zero-order kinetics. The velocities of the reaction with each enzyme system were measured at 1, 300, 600, and 1000 atm and at 4°, 12°, 22°, and 30°C. Although all three enzyme systems exhibited some phosphatase activity even when compressed to 1000 atm at 4°C, the rate was retarded by both compression and by refrigeration almost as a straight-line function of the log of the temperature and pressure. The unexpected pressure tolerance of the enzyme from the calf intestine may be explained

by the existence of an equilibrium between active and inactive enzyme, a problem which merits much more attention. Detailed data and a discussion of the significance of these experiments will be the subject of a paper that is being prepared for publication.

Mr. Palmer has continued to examine the effect of compression on the mutation rate of Serratia marnorubra to streptomycin resistance. A log phase culture is dispersed in nutrient broth to give a concentration of 50 to 500 viable bacteria per ml. This inoculated broth is dispersed in several dozen 2.5-ml test tubes, sealed with neoprene piston-type stoppers, for incubation at different pressures at 30°C. After incubation from 0 to 8 hours, the entire contents of each replicate tube are transferred to tubes of nutrient broth treated with streptomycin sulfate to give 125 µg/ml. If one or more mutants to streptomycin resistance have developed, with further incubation growth will occur in the streptomycin broth. In some experiments, the mutation rate at 300 atm was several hundred times greater than at 1 atm. Cultural tests demonstrated that the observed effects were not due to a killing of streptomycin-sensitive cells by pressure. It was also demonstrated that oxygen tension, which is difficult to control under conditions of compression, was not responsible for the greatly increased mutation rate observed in compressed cultures. The average mutation rate found at 1 atm was 1.7×10^{-8} per bacterial division as compared with an average rate of 0.95×10^{-8} at 300 atm.

PLANS FOR FUTURE

Although the ONR Contract under which this work has been done expires with this report, plans are being made to continue investigating the multiple effects of increased pressure on the behavior of bacteria. Most promising is work currently in progress on the effects of pressure on the temperature tolerance of microbes, the study of pressure as a mutagenetic agent, and the explanation of differences in the effects of pressure on different organisms. At hand are data for three or four papers, which are now being prepared for publication.

CURRENT REPORTS AND PUBLICATIONS

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- (b) C. E. ZoBell (1959), "Introduction to marine microbiology." *New Zealand Oceanogr. Inst. Mem. No. 3*, 7-23.
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- (d) C. E. ZoBell (1959), "Thermal changes accompanying the compression of aqueous solutions to deep-sea conditions." *Limnol. Oceanogr.* 4, 463-471.

15 December 1959

SPORE FORMATION AND SPORE GERMINATION
IN BACTERIA

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ASSISTED BY (Part time) - P. H. Hodson, H. Foester
(Full time) - Dr. L. J. Rode, Eliz. Matthews

TASK NO. NR 103-025

CONTRACT Nonr-146(00)

OBJECTIVES

To study the fundamental biological and biochemical processes in microorganisms.

ABSTRACT

A number of experimental approaches were designed to elucidate the function of dipicolinic acid (DPA) in bacterial spores through the location of the DPA, and to determine the nature of the spore state. Mechanical rupture of spores of Bacillus megaterium released practically all of the DPA in free form. Boiling spores for a few minutes released all of the DPA. Combinations of mild heat (56°C) and certain concentrations of selected solvents, H₂O₂, phenol or surface active agents released DPA very efficiently. About 30 per cent of the dry weight of the spores is lost, the other major component extracted being one or more mucopeptides. In a number of respects the surfactant treated spores resemble germinated spores, except that they are not viable. They are stainable and nonrefractile. DPA is removable from spores of B. megaterium by electrodialysis. Shaking spores in the presence of powdered glass causes changes in the spores that are practically identical with those of physiological germination. The abraded spores are viable, nonrefractile, stainable, heat sensitive and have lost dipicolinic acid and mucopeptides.

ISOLATION OF TRANSFORMYLASE AND PTEROICASES A AND B FROM
ESCHERICHIA COLI

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University of Pennsylvania

ASSISTED BY T. Yokota

TASK NO. NR 103-045

CONTRACT Nonr-395(00)

OBJECTIVES

Isolation and study of transformylase and pteroisases for the enzymatic differentiation of sulfathiazole-sensitive and sulfathiazole-resistant strains of E. coli.

ABSTRACT

The outstanding discovery in our investigation is that the ST-sensitive strain contains only the ST-sensitive pteroisase A. In contrast, the ST-resistant strain contains two pteroisases. One, ST-sensitive pteroisase A and a second one, the ST-resistant pteroisase B. This finding is of great significance in understanding the mechanism of the development of resistance to ST.

The isolation of transformylase and pteroisases from E. coli was carried out by harvesting the cells at 50% of the optimal growth, washing thoroughly with 0.66 M phosphate buffer of pH 7.3, sonicating for 30 minutes, and centrifuging. The clear supernate was treated with ascorbic acid to a molarity of 0.04 M to 0.005 M. After overnight refrigeration it was 20 to 40% saturated with ammonium sulfate. The precipitated protein was dissolved in 0.066 M phosphate buffer of pH 7.3 and dialyzed overnight against distilled water in the cold room. It was then dialyzed twice in succession in the cold room each time against 200-fold volume of 0.033 M phosphate buffer of pH 7.38. It was then purified using alumina chromatography followed by Dowex 50 resin column chromatography. The eluate from Dowex 50 column contained highly purified transformylase and pteroisase A. Pteroisase A from both the ST-sensitive and ST-resistant strains is inhibited by ST. Transformylase also has inosinase activity.

The fraction obtained from the ST-resistant strain by 60 to 100% saturation with ammonium sulfate and by further purification outlined above yielded pteroisase B which is not inhibited by or is resistant to ST. ST-sensitive strain did not yield pteroisase B under these conditions. It would seem that during the development of resistance to ST, ST-resistant pteroisase B is produced. This enzyme is either a new entity or is obtained from the ST-sensitive pteroisase A during the development of resistance to ST.

PLANS FOR FUTURE

Studies are in progress to determine the exact stage at which

pteroicase B appears during the process of the development of resistance to ST. We are also experimenting to determine whether or not ST-sensitive pteroicase A can be eliminated by increasing the resistance to ST.

LOBUND GERMFREE LIFE RESEARCH LABORATORIES
PHASE I: MORPHOLOGY AND PHYSIOLOGY

H. A. Gordon
University of Notre Dame

ASSISTED BY T. Staley and E. Bruckner-Kardoss

TASK NO. NR 103-067

CONTRACT Nonr-i523(04)

OBJECTIVES

(a) To establish physiological and morphological standards for the understanding and use of germfree animals in biological and medical experiments, (b) to study the effects of the normal flora on the animal host, and (c) in extramural collaborations, to study the relations of bacterial flora to irreversible shock, to compare immune responses of germfree and conventional animals and to study the effects of total body X-irradiation in germfree and conventional mice.

ABSTRACT

(a) Previous studies demonstrated that germfree chickens displayed satisfactory criteria of normality. Until recently, however, the germfree rat showed retarded growth, reduced reticulo endothelial tissue throughout, adrenal enlargement and the cecal enlargement characteristic of germfree rodents. Mice were in most respects similar to the germfree rats. In 1959 efforts centered on determining the relative contributions of the absence of bacteria and of other non-specific stress factors to these anomalies in the germfree rat. Non-bacterial factors studied included relief from crowding, surgical removal of the enlarged cecum and the effect of the tank-type environment on conventional rats. Observation of germfree rats provided increased living space, others cecectomized and conventional rats maintained in Reyniers tanks, warrant the present conclusions: underdevelopment of RE elements in germfree rat comparable to germfree chicken; increased floor space per germfree rat seemed to minimize or eliminate previously observed anomalies associated with non-specific stress; the enlarged cecum is not a "secondary stressor agent"; the germfree rat now exhibits a considerable degree of structural and functional "excellence"; and, finally, the tank type environment itself is not responsible for the stress anomalies previously associated with germfree rats. The search for bacterial products capable of reducing the enlarged cecum involved devising a semipermeable nitrocellulose capsule capable of holding bacteria while permitting diffusion of their products through the capsule wall. The intention was to determine if the bacterial products released by a capsule inserted into the lumen of the cecum through a fistula would reduce the cecum. Difficulties in sterilizing capsule surface and in neutralizing cellulose digesting bacteria were insurmountable. A parallel program to identify, if possible, "cecum-active" bacteria by monocontamination of originally germfree rats has just been started. (b) Previous study of the effects of the normal flora on the gut tract of the host has revealed that in all species investigated the presence of the flora increased the mass and hydration of the intestinal wall as well as the RE cell population. This year the study of the small intestine was extended to measuring tissue

distribution, mitotic activity and total mucosa surface. A further elaboration of the physiological flora effects also includes transfer of water, Na^+ and K^+ across the intestinal membrane in germfree and conventional rats and flora effects on tonus of isolated rat intestine along with the continuing study of the effect of oral antibiotic treatment in conventional rats. In the lamina propria the germfree chicken manifested its lack of bacterial stimulation by a significantly lower percent tissue distribution. Monocontamination with Cl. perfringens or Str. faecalis raised the lamina propria and core of villus percent tissue distribution to values approximately midway between germfree and conventional data. Penicillin treatment of both monocontaminated and conventional animals maintained the lamina propria and core of villus values essentially at germfree levels. Higher RE cell count and increased hydration on bacterial stimulation of the lamina propria contribute to part of the tissue increment observed but not to the extent necessary to account for the total increment. Actual proliferation of the connective tissue framework appears to be involved. Preliminary use of the colchicine method to count the number of cells in arrested mitotic activity in the villus and Lieberkuhn epithelium in the lower small intestine of 100-200 days old germfree and conventional rats led to such variability of results that no pattern is yet discernible. It does appear, however, that in both the germfree and conventional animals the bulk of mitotic activity of the small intestine's epithelium takes place in the Lieberkuhn krypts. The histological reconstruction method of Fisher and Parsons applied to specimens of small intestine from 98-102 days old germfree and conventional rats revealed a marked decrease of the total mucosal surface of the small intestine of the germfree rat compared to the conventional. Also the average villus of the mid and lower small intestine of the germfree rat appeared shorter and more slender than that of its conventional counterpart. Flora effects on intestinal absorption (involving only conventional rats so far) are being determined by observing the transfer of water across the intestinal membrane as established by the change of phenol red concentration in the intestinal perfusate. Na^+ and K^+ are determined by flamephotometry in the same medium. Also in the preliminary stage is kymographic analysis of the effects of standardized physiological stimuli on isolated muscle strips. The fact that oral administration of penicillin at nutritional levels had previously been observed to impart "germfree like" characteristics to conventional chickens prompted a comparable experiment with conventional rats. Except for a significant deficit in the submandibular lymphoid tissues, the treated and untreated conventional rats were substantially alike. No cecal enlargement nor any other "germfree characteristics" were observed in the animals fed penicillin. (c) Previous collaborative research with Zweifach and his associates had failed to uncover any significant difference between the susceptibility of germfree and conventional rats to irreversible hemorrhagic shock. This year, germfree and conventional animals subjected to the shock produced by temporary occlusion of the superior mesenteric artery were compared. The germfree rat proved to be somewhat more susceptible to this form of intestinal ischemia than the conventional controls. No blocking action of the adrenergic drug, dibenzylamine, could be observed in the germfree animal. Cecectomy of germfree rats did not affect their susceptibility to either hemorrhagic or SMA occlusion shock. Cecetomized germfree rats seeded orally with E. coli several weeks before the shock treatment showed no difference from conventional in either types of shock. Using proferrin as a blocking agent for the RE system, germfree rats were thereby

predisposed to lethal shock. This evidence points to some mechanism other than increased susceptibility to bacterial endotoxins that block the RE system in experimental shock. The fact that depression of the RE system and histologic changes occur in germfree rats following shock suggests that factors other than bacteria are responsible for the lethal course of protracted shock. A further collaboration with Speirs failed to show any significant difference between conventional and germfree rats in their cellular and antibody responses to primary and secondary injections of tetanus toxoid. Just begun in collaboration with Smith (NIH) are studies of the effects of total body irradiation of germfree and conventional mice with special reference to survival and response to administration of bacterial endotoxin.

PLANS FOR FUTURE

(a) The need for continued broad testing and characterization of germfree animals is coming to an end. New species will have to be tested as will the effects of new environments such as plastic isolators. The "enlarged cecum" problem of the germfree rodent will be pursued further. Also work on the "isolator conventional" animal. (b) Surface mucosa determinations in germfree chickens and in antibiotic treated animals will be made. A lead suggesting that flora may affect capillary blood supply to some organs will be followed up. In vitro preparations of intestinal strips may be used in a screening program to study effects of microorganisms and their products on intestinal tonus and motility. (c) The three collaborations will continue.

CURRENT REPORTS AND PUBLICATIONS

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(b) H. A. Gordon (1959). "Morphological and Physiological Characterization of germfree life." *Ann. N.Y. Acad. Sci.* 78, 208-220.

(c) H. A. Gordon (1959). "The use of germfree vertebrates in the study of 'physiological' effects of the normal microbial flora." *Gerontologia*, 3, 104-113.

(d) H. A. Gordon, M. Wagner, T. D. Luckey and J. A. Reyniers (1959). "An encephalomeningical syndrome selectively affecting newly hatched germfree and monocontaminated chickens." *J. Infect. D.*, 105, 31-37.

(e) H. A. Gordon, (1959). "Historical aspects of germfree experimentation." *Proc. II Symp. Gnoto. Tech.* 9-18.

(f) H. A. Gordon, B. S. Wostmann and M. Wagner (1959). "The role of the microbial flora in maintaining 'physiological levels' of reticulo-endothelial cells, serum proteins and circulating antibodies." *XXI Int. Cong. of Phys. Sci.*, Buenos Aires, 110-111.

LOBUND GERMFREE LIFE RESEARCH LABORATORIES
PHASE II: BACTERIOLOGY, SEROLOGY AND IMMUNOLOGY

M. Wagner
University of Notre Dame

ASSISTED BY E. Werderitsh

TASK NO. NR 103-067

CONTRACT Nonr-1623(04)

OBJECTIVES

(a) To study: the effects of monocontaminating chickens with Cl. perfringens or Str. faecalis; the possible role of bacteria in heart valve granulations observed in monocontaminated chickens; and the bacteriology of the alimentary tract of conventional rats confined in a Reyniers-type isolator. (b) To determine: response of the rat to parenteral purified antigen; serological aspects of monocontaminating chickens, some fed penicillin, with Cl. perfringens and Str. faecalis; agglutinin titers. (c) Dental caries, periodontal disease and bacteriology of shock.

ABSTRACT

(a) Bacteriological aspects of the monocontamination of chickens with Cl. perfringens or Str. faecalis are included in Phase III: Biochemistry and Nutrition. Earlier observations: chickens contaminated after a period of germfree life revealed heart valve granulations. Current efforts are directed toward reproducing this syndrome through selective monocontamination of germfree chickens. Results are not yet available. So-called "locked flora" effect created by confinement of conventional rats in a Reyniers-type isolator operated exactly as if it were germfree is now under preliminary study to determine whether rats so confined differ microbiologically from rats reared in the usual open animal room. (b) Wostmann showed germfree rat serum to have alpha -2, beta and gamma globulin levels lower than conventional rat serum. All these depressed fractions in germfree rats increased after the rats were placed in a conventional environment. To relate these changes to an antibody response, germfree and conventional rats were injected parenterally with a purified antigen, bovine serum albumin (BSA). While a single dose of BSA produced a precipitin titer of 1:16,384 in the chicken, no response was demonstrated in the rat. Freund incomplete adjuvant was then used in an attempt to stimulate in the rat an immunological response to BSA without success. Further tests indicated that the rat sera did not seem to contain a blocking antibody for the BSA antigen absorbed on tanned chicken and bovine erythrocytes. Agglutinin titers for Micrococcus epidermidis have appeared in the serum of later generation germfree colony rats fed diet L-356. Appearance of these agglutinins seemed to coincide with the change from a loose granular diet to sterilized pelleted diet of the same composition thus raising the possibility that pelleting preserves some of the bacterial antigens in the sterilized diet. Comparison agglutinin titers of germfree rats fed granular diets with those fed pelleted diets was inconclusive because of the variability and inconsistency of the results. (c) Emergence of caries resistant rats from the Lobund strain required selection and breeding of a caries susceptible line to keep the research progressing. Such a line seems to have been established during

1959. Germfree rats monocontaminated with Str. faecalis and fed a cariogenic diet developed dental caries. Similar experiments using a strongly acidogenic Lactobacillus are now underway. A group of rats in their 15th month maintained on a "cariogenic type" diet are being observed for development of periodontal disease. Attempts were made to follow tissue invasion by bacteria following a hemorrhagic or SMA-type shock in conventional monocontaminated and germfree rats. Experimental conditions tended to obscure the relative roles of bacterial invasion and surgical contamination during surgery to induce shock in conventional rats.

PLANS FOR FUTURE

(a) The role of bacteria in the heart valve granulation syndrome observed in chickens will be pursued. Attempts will be made to reproduce the syndrome in other germfree species. Possibility of reversing the syndrome by further selective contamination will be explored. Enough data on the "locked flora" effect will be accumulated to demonstrate whether it is significant. (b) Immune response to BSA will include check of "cryptagglutinoids" by a modified indirect Coombs technique for rat serum protein. Studies of serological and immunological effects of monocontaminating chickens with Cl. perfringens will continue. More Lactobacillus monocontaminated rats are being bred to repeat the agglutinin titrations using this organism. Since the original observations were inconsistent, new groups of rats, some on pelleted others on granular diets, will be studied. (c) The extramural collaborations on dental caries, periodontal disease and bacteriological aspects of shock will continue.

CURRENT REPORTS AND PUBLICATIONS

- (a) M. Wagner and B. S. Wostmann (1959). "Studies on monocontaminated chickens (Clostridium perfringens or Streptococcus faecalis) fed penicillin." Antibiotics Annual, 1958-59, 1003-1011.
- (b) M. Wagner (1959). "Serological Aspects of Germfree Life." Ann. N.Y. Acad. Sci., 78, 267-271.
- (c) M. Wagner (1959). "Determination of Germfree Status." Ann. N.Y. Acad. Sci., 78, 89-101.
- (d) M. Wagner (1959). "Determination of Germfree Status." Proc. II Symp. Gnoto. Tech. 83-95.

LOBUND GERMFREE LIFE RESEARCH LABORATORIES
PHASE III: BIOCHEMISTRY AND NUTRITION

B. S. Wostmann
University of Notre Dame

ASSISTED BY L. Knight, J. Pleasants and G. Olson

TASK NO. NR 103-067

CONTRACT Nonr-1623(04)

OBJECTIVES

(a) In biochemistry to study: histamine and serotonin levels in the intestinal tract of germfree and conventional rats; immuno-electrophoretic analysis of germfree rat serum; effects of the presence of a controlled bacterial flora; and the effects of the stimulation of germfree rats with an antigen. (b) In nutrition to study: utilization of flora-synthesized vitamin B₁ by the host; serum cholesterol levels in germfree animals; action of vitamin K; nutrition of germfree rats. (c) As an adjunct to germfree rearing and breeding, to study rearing of animals delivered by Caesarian into sterile environments.

ABSTRACT

(a) The amines, histamine and serotonin, seem to play a role in regulating capillary blood supply and phagocytosis. Histamine values in the lower intestine of the germfree rat were much lower than in the conventional rat; there was no significant difference in comparable data for germfree and conventional chickens. Little difference was noted in serotonin levels in lower intestinal walls of germfree and conventional rats and chickens. Histamine appears to be one of the amines involved in regulating one or more critical phases of the host defense mechanism of the rat. Immuno-electrophoretic technics by P. Grabar (Paris) using anti-rat (conventional) and anti-mouse (conventional) rabbit immune serum shows germfree rat and mice sera to have the same fractions as conventional controls. This technique also demonstrates the marked difference in some immuno-electrophoretically detected fractions of germfree and conventional rat serum. Preliminary electrophoresis of rabbit serum shows a significantly lower gamma globulin level in the germfree compared to the conventional rabbit. Young conventional chickens fed penicillin at nutritional levels from birth display a morphology unlike conventional but qualitatively similar to germfree birds. Although penicillin feeding does not reduce the total viable count of intestinal organisms, the Cl. perfringens population is markedly reduced while Str. faecalis count in the lower ileum also seems lower. This prompted penicillin feeding to chickens monocontaminated with Cl. perfringens or Str. faecalis. Cl. perfringens as monocontaminant intensely stimulated the RE system of the intestinal wall but generated only slightly higher gamma globulin levels than seen in germfree chickens. Agglutinating antibodies were at a low level. Str. faecalis as monocontaminant resulted in high (near normal) gamma globulin and antibody titer levels along with only moderate stimulation of the cellular defenses. Addition of 50 mg/kg procaine-penicillin G to the diet returned lamina propria tissue to germfree levels with Cl. perfringens count drastically reduced or Str. faecalis count hardly at all. Thus the tissue response seems not to depend on numbers of organisms but rather on some change in its metabolism effected by the antibiotic. As noted in Phase II, to confront germfree animals with a

complicated antigenic stimulus, germfree rats were inoculated with Bovine Serum Albumin (BSA). Not until BSA was combined with Freund's incomplete adjuvant was a response in the beta fraction of the serum protein pattern detectable. No anti-BSA circulating antibodies could be demonstrated, however, using precipitin test, tanned erythrocyte technic or test for blocking antibody. (b) Previous evidence showed conclusively that S^{35} is incorporated into the thiamine molecule from $S^{35}O_4$ by the intestinal flora of the conventional rat. This year, radio sulfate injected directly into the cecum increased the specific activity of the flora-synthesized thiamine. The amount of flora-synthesized thiamine the rat could take up was less than one microgram per day. Analysis of intestinal contents of both germfree and conventional rats once again pointed to the cecum as the main site of bacterial synthesis. Although thiamine is not bound to larger cell fragments after centrifugation at 40,000 rpm for 15 minutes, nevertheless absorption from the cecum or large intestine does not take place. Serum cholesterol levels in germfree rats on L-356 and L-462 diets appear slightly lower than in conventional rats. Since bodyweight of germfree rats tends to be slightly lower than of conventional rats, these results are somewhat inconclusive. Cl. perfringens monocontamination of chickens increases cholesterol level by 50% substantiating work by Kritchevsky pointing to the possible role of intestinal flora in cholesterol and bile acid metabolism. In studying the role of vitamin K in the homeostatic and hemostatic mechanisms, attempts were made to produce a diet absolutely devoid of vitamin K. When fed a non-extracted standard diet with vitamin K omitted, germfree rats manifested no signs of "hemorrhagic syndrome from juvenility to adulthood. A non-extracted diet stripped of possible vitamin K contaminant gave the same result. An extracted diet carefully tested for total absence of vitamin K fed to germfree rats resulted in death through hemorrhage in 10 to 15 days. Two of twelve conventionals died similarly while the others showed signs of deranged clotting mechanism. Hemalogical analysis of the germfree animals revealed abnormalities apparently not related to vitamin K deficiency. The remarkable alterations of the hematopoietic system under these experimental conditions is not depicted in the literature and suggests a reevaluation of vitamin K's role vis a vis other nutritional factors. Further studies on the distended cecum of the germfree rats developed data indicating that the low dry matter content in the cecum is "normal" for the germfree animal; low dry matter content does not necessarily mean cecal distension; and a lack of flora-produced histamine does not lead to cecal distention. Monocontaminated rats have not shown much, if any, sign of cecal reduction. A study was run on a modification of the Grienstein-Birnbaum low molecular, water soluble diet for handfeeding baby rats. (c) Handfeeding of rats on the low molecular, water soluble diet was attempted to get around antigenic stimulation of the host by large protein molecules, killed bacteria, etc. While some progress was made, a fully effective diet and feeding technique remains to be developed. The germfree rabbit rearing program concentrated on improvement of diet, removal or ligation of cecum to prevent volvulus and the rearing of Dutch strain rabbits with their favorable characteristics of small size and early maturity.

PLANS FOR FUTURE

(a) Germfree rats will be monocontaminated to study changes including histamine levels, as a function of time; immuno-electrophoresis methods will be used to follow changes in serum proteins following monocontamination; serum patterns of rabbits, mice and guinea pigs will be

observed. The BSA response of germfree rats will be restudied. (b) Paper electrophoresis to examine coupling of thiamine to protein materials; further study of the effects of selected monocontaminants or cholesterol levels; changes in blood coagulation factors and hematology of bone marrow and peripheral blood; effects of challenging germfree rats with various bacterial toxins. (c) Further refinement of low molecular, water soluble diet for handfeeding; continuation of handfeeding and rearing programs for rats, mice and rabbits.

CURRENT REPORTS AND PUBLICATIONS

- (a) Bernard S. Wostmann, P. Leonard Knight and J. A. Reyniers. The influence of orally-administered penicillin upon growth and liver thiamine of growing germfree and normal stock rats fed a thiamine deficient diet. *J. Nutrition*, v. 66, no. 4, December, 577-586.
- (b) Bernard S. Wostman. "Serum proteins in germfree vertebrates. *Annals of the N.Y. Acad. of Sci.*, vol. 78, Art. 1, pp. 254-260, May 8, 1959.
- (c) Bernard S. Wostmann. "Nutrition of the germfree mammal. *Annals of the N.Y. Acad. of Sci.*, vol. 78, Art. 1, pp. 175-182, May 8, 1959.
- (d) Bernard S. Wostmann and Julian R. Pleasants. "Rearing of germ-free rabbits. *Proc. of Animal Care Panel*, June, 1959, vol. 9, No. 2, p. 47.
- (e) Bernard S. Wostmann and Edith Bruckner-Kardoss. "Development of cecal distention in germfree baby rats." *Am. J. of Physiology*, 197:1345, 1959.

LOBUND GERMFREE LIFE RESEARCH LABORATORIES
PHASE IV: GNOTOBIOTICS

P. C. Trexler
University of Notre Dame

ASSISTED BY C. K. Smith and R. Hakes

TASK NO. NR 103-067

CONTRACT Nonr-1623(04)

OBJECTIVES

(a) To develop the various components of closed system contamination control apparatus and (b) to apply closed systems to various operations including the rearing and using of gnotobiotics.

ABSTRACT

(a) Contamination rates and direct operational costs were determined for thirty-five weeks of operation of an average of 8.9 isolators per week for breeding and holding rats and 4.8 isolators per week for breeding and holding mice, i.e., 13.7 isolators/week for 35 weeks or 479.5 germfree isolator weeks. Contaminations experienced totaled 5 for an average of 1 per 95.9 isolator weeks or 1% per isolator week. Operation of these isolators required 84.7 man hours/week. Amortizing equipment over 10 years on a straight line basis the yearly cost of operating these colonies was \$14,478. The colony produced at the rate of 1560 mice/year and 348 rats/year. Cost was \$7.89 per germfree animal including the cost of holding the animals for disposition. The colony was primarily used to test commercial rations. The production achieved was about half that attained on Lobund synthetic diets. The jacket isolator designed to increase germfree production potential by incorporating a flexible protective garment in an air supported plastic bag showed great promise in its first 13-week run terminated by a mechanical failure. It is being developed in collaboration with Levenson (WRAIR) for special applications in human surgery and for patient treatment. During 1959, 761 germfree mice and 137 germfree rats in 24 separate shipments were sent to other laboratories by station wagon, rail or air express. Only two shipments were contaminated. (b) Breeding colonies of mice and rats were maintained in flexible film isolators from which 1619 mice and 250 rats were weaned. A colony of C57 mice has been started to supplement the Webster-Swiss stock (Lobund strain). To facilitate gnotobiotic experiments with their associated inherent limitation on the use of litter mate controls, inbred lines will be developed. Of the five contaminations experienced, only two could be associated with the development of the chemically sterilized plastic isolators. The other three involved gloves (2) and a trap malfunction, features common to all isolators. A steam sterilizeable commercial ration was used in the colony. The commercial ration proved deficient yielding only half the weanlings produced by semi-synthetic diet L-462 prepared in the Lobund diet laboratory. Clyde K. Smith successfully delivered a germfree sheep

into a sterile plastic isolator on December 3, 1959 and at year's end it was still germfree. It took 24 days to gain 5 lbs. compared to 32 days for a conventional sheep also housed in a plastic isolator and fed the same diet.

PLANS FOR FUTURE

(a) A series of jacket isolators (as many as four) will be put into operation for breeding and rearing germfree rats and mice. Operational and cost data will be compiled. Evaluation for use in human surgery in collaboration with WRAIR will be continued. Inbred colonies suitable for gnotobiotic experiments will be started and maintained. Shipment techniques, particularly using public transportation will be further developed. (b) Effects of selective bacterial monocontamination on germfree rats and mice will be studied on a systematic organism screening basis as the large production of jacket isolators becomes available. Particularly, a search will be made for an animal with a defined, non-pathogenic flora suitable for experimentation but free of the physiological anomalies seen in germfree rodents.

CURRENT REPORTS AND PUBLICATIONS

- (a) Philip C. Trexler (1959). "Progress Report on the Use of Plastics in Germfree Equipment." Proc. Animal Care Panel, 9, 119-125.
- (b) P. C. Trexler (1960). "Introduction to the Symposium." Proc. 2nd Symp. Gnoto. Tech., 1-7.
- (c) P. C. Trexler (1960). "Flexible-wall Plastic Film Isolators." Ibid., 55-60.
- (d) P. C. Trexler (1960). "Sterile Rooms." Ibid, 121-125.
- (e) P. C. Trexler (1960). "Gnotobiotics in Relation to Space Biology." Proc. Soc. Ind. Microbiol. (In press.)

LOBUND GERMFREE LIFE RESEARCH LABORATORIES
PHASE V: VIROLOGY

T. G. Ward
University of Notre Dame

ASSISTED BY E. Kamphausen, L. Lindholm and A. Burns

TASK NO. NR 103-067

CONTRACT Nonr-1623(04)

OBJECTIVES

(a) To study the effects of monocontamination of germfree mice with known viruses; (b) to determine whether the germfree animal is virus free, especially with respect to masked agents; (c) to study the effects of burns on germfree animals; (d) to collaborate on a histological survey of germ-free rats; (e) to study the occurrence of spontaneous tumors in germfree animals.

ABSTRACT

(a) Of interest was the LD₅₀ of PR8 influenza virus required for germfree as compared to conventional mice. Lack of sufficient mice of 15-17 grams prevented completion of this study. Live PR8 virus in chick allantoic fluid was given to germfree male mice with a marked delay in reaction (ONR 1958). In 1959, protocol was repeated. Seven days after vaccination with 0.5 ml of live PR8 virus in allantoic fluid, bacteriologically sterile, hemagglutination inhibition titer for germfree mice was less than 2 and for normal mice was 128. A second dose at 7 days caused germ-free titer to increase rapidly indicating that the defense mechanism of the germfree mouse was "alerted" by the first dose of PR8 antigen and then responded to the second dose substantially in the same fashion as the inoculated conventional mice; (b) Successive passages at seven day intervals within the germfree system of ground germfree rat lung resulted in lung consolidation at about the fourth or fifth passage increasing to involve about 50% of the lung at the tenth or eleventh passage. In one run this consolidation persisted to the 19th passage. Eight protocols resulted in substantially the same results. All ended in contamination but passage was continued in the presence of the monocontaminants with no appreciable change in the degree of consolidation indicating no synergistic effect of the bacterial contaminant on the consolidating agent. This "gray lung virus" is not yet definitely characterized as an actual virus agent. Although pneumonia virus of rats was easily demonstrated in the conventional control colony, standard procedure failed to indicate its presence in the germfree rat colony. Tissue culture of six germfree rat lungs in mouse embryonic tissue and serum of 18 germfree rats failed to reveal the presence of RV virus. Also salivary gland virus could not be demonstrated using tissue culture and serological methods in 14 germfree rats and 11 germfree mice. Serological testing for various viruses in the serum of 27 germfree mice and 16 germfree rats failed to uncover polyoma, FL, K, salivary gland of Gross leukemia viruses at least in concentrations sufficient to induce demonstrable antibodies. The conventional colony shows polyoma, salivary gland and possibly, Gross leukemia. After 3 years testing the

limited evidence available tends to support the conclusion that Lobund's germfree mice are free of demonstrable viruses and the germfree rats may only have one--a "gray-lung agent" yet to be characterized. (c) LD₅₀ for germfree and normal mice burned after anesthesia in a 70°C water bath for varying length of times was determined. Typical results for 16 germfree mice, 20.5 gms average weight showed LD₅₀ = 6.4 seconds; for 20 normal mice, average weight 20 gms, LD₅₀ = 8.8 seconds. Throughout the weight ranges (15-30 gms) tested, more heat was required to kill normal mice than germfree of comparable weight. The difference diminished as the weights increased, however. Upon autopsy, burned germfree mice showed hyperemia and engorgement of the lungs whereas the lungs of normal mice appeared normal. On the other hand, the mesenteric area of the normal mice showed engorgement and that of the germfree appeared normal. Germfree and normal rats were burned under anesthesia on a hot plate for various times at different degrees of heat. No difference in the He La cell growth inhibition capability of the burned germfree and burned normal rat sera was demonstrable. This failure to demonstrate a toxin may reflect a lack of quantitation in the tests. After burning by immersion, fluids from an "air sac" created by subcutaneous injection of 30 ml of air between the shoulder blades of germfree and normal rats were collected, pooled and lyophilized for subsequent analysis (Dr. Rosenthal). When lyophilized, the secretions from normal animals were light gray, amorphous and in relatively large amounts. The secretions from germfree animals were much darker, more granular and collectable in less quantity. These gross observations suggest some substantial difference in the composition of the secretions from germfree and normal rats. (d) A histological survey of ten germfree and ten conventional rats ranging in age from 58 to 541 days has been completed in a collaboration with R. Wissler and associates. Consistent differences were noted in a preliminary report. (e) Germfree animals offer a unique opportunity to study the relation between tumors and viruses since they are reared in the absence of demonstrable bacteria, yeast, molds, fungi, parasites and rickettsia. Although the absence of viruses cannot be stated with the same degree of certainty, tissue culture and immunological methods have failed to demonstrate pneumonia virus of mice, salivary gland, "K" and "FL" virus, polyoma, Gross leukemia, hepatitis, mouse encephalomyelitis (Theiler's) and lymphocytic choriomeningitis. A statistical comparison of the occurrence of spontaneous tumors in the germfree and normal colonies is being initiated on IBM cards. C₃H, C₅₇ black and DBL strains of mice are being started germfree and comparable records will be kept. Upon detection of a tumor, the germfree animal is sacrificed, serum collected, tumor excised, part for histological samples, part for tissue culture and part ampouled and frozen at about -70°C. Establishment of continuous cell lines for the tumor is attempted in tissue culture. Cells from the tumor are passaged to young germfree animals and possible "takes" observed. Tissue culture within the germfree system is being tried to overcome objections of virus contamination from the culture medium. Serum is tested against a battery of viruses both at Lobund and at NIH (Huebner). Eleven spontaneous tumors have been studied, nine from germfree rats and two from the conventional colony. Three tumors from mice (1 germfree) have also been studied. Continuous cell lines have been established for 8 of 14 specimens (5 germfree and 3 normal). Tissue culture cells secured from a spontaneous tumor of a germfree rat produced a tumor in a germfree rat upon inoculation.

PLANS FOR FUTURE

(a) An experiment to determine how the "alerted" reticulo-endothelial system of PR8 injected mice if a second antigen (such as Lee influenza) is injected at around 7 days will be run as soon as the germfree animal supply permits. (b) Studies to characterize the "gray lung agent" will continue; other protocols will be repeated to provide a more reliable statistical base for conclusions about the viral status of germfree rats and mice. (c) Complement and hemagglutination procedures using red blood cells will be applied to sera of burned animals; (d) Histological survey will continue with comparison of globulin levels and "plasma" cells and comparison of germfree animals at various ages. (e) Observation and testing of spontaneous tumors in germfree animals will continue; tumor producing viruses such as papilloma, sarcoma 37 and 180, or leukemia will be tried in germfree animals as well as tumor producing chemicals. Interaction of known tumor producing viruses and other microorganisms will be studied in germfree animals.

CURRENT REPORTS AND PUBLICATIONS

(a) Thomas G. Ward and Louise Lindholm (1959). "Effects of Burns in Germfree Animals." Report to Bureau of Medicine and Surgery, Department of the Navy, Oct. 2, 1959.

LOBUND GERMFREE LIFE RESEARCH LABORATORIES
PHASE VI: MEDICAL BACTERIOLOGY

Rev. James P. Doll, C.S.C.
University of Notre Dame

ASSISTED BY

TASK NO. NR 103-067

CONTRACT Nonr-1623(04)

OBJECTIVES

(a) To study: the pathogenesis of "L"-forms of bacteria; (b) phagocytosis in germfree animals; (c) helminth infections in germfree animals; (d) clearance of colloidal carbon from the blood of germfree rats following hemorrhagic and SMA shock.

ABSTRACT

Previous work showed that massive doses of GL 4817 and GL 4829 both derived from Group A beta hemolytic streptococci were not pathogenic to conventional mice. Accordingly, isolation of the more virulent L-form of Pseudomonas fluorescens was attempted without success; L-forms of the mouse pathogenic Smith strain have been obtained but, as yet, they have not been adapted to broth culture. (b) In vitro work previously used as the criterion of phagocytosis the ability of circulating polymorphonucleated neutrophils to phagocytose bacteria. This work with germfree animals is now extended to the methods of Benaceraff et al for carbon and labeled bacterial clearance in vivo. Initially, phagocytic ability of circulating polymorphonucleated neutrophils was shown to be nil in germfree animals. Subsequently, both positive phagocytosis and circulating antibody were found. This new result coincided with a change from granular to pelleted diet in the colony raising the possibility that the pelleted form offered an increased antigenic stimulus to the germfree animals. To test this hypothesis two groups of germfree rats, one on pelleted and the other on non-pelleted diet, were tested for phagocytic index and for agglutinating antibody. Phagocytic index for both groups remained high. Water added to the granular diet caused clumping, however, so that it may have been comparable to the pelleted diet. Carbon clearance for germfree and conventional mice was essentially the same. A significant difference between germfree and control mice was observed for clearance of E. coli but not for Staphylococci. (c) Embryonated eggs of Heterakis gallinae harboring the protozoa, Histomonas meleagridis were sterilized without loss of viability by 4 minute immersion in 0.5% solution of peracetic acid. Eggs fed to conventional turkeys were highly infective with typical death rates and post mortem pathology. Fed to germfree turkeys, there were very few "takes", few worms survived and liver lesions were noted in only one of seven birds. Monocontamination with unspecified micrococci occurred so that the run cannot be considered germfree. (d) Carbon clearance was used as an index of recovery of germfree and conventional animals from hemorrhagic and SMA shock in collaboration with Zweifach et al. Measurements were made prior to shock and after shock on

survivors. Control clearance rates of germfree and conventional rats prior to shock were essentially the same. Study of 6 survivors of shock, 5 showed a significantly lowered clearance rate while one exhibited an accelerated rate.

PLANS FOR FUTURE

(a) McCarty has successfully grown L-forms on Bray dishes. Isolation of *Pseudomonas* and Smith strain will be tried using this method; (b) statistical baseline will require testing more conventional animals using P32-labelled cocci and coli organisms. Similar indices will be established for germfree rats and a standard K-value derived for them. (c) The Hetarakis infection of germfree turkeys will be repeated. If lesions can be produced in bacteria-free conditions, attempts will be made to procure *Histomonas* in pure culture and to raise it axenically in vitro. If lesions cannot be produced under sterile conditions, a monocontaminant symbionts will be studied. Raillietina cesticillus, a gut parasite of chickens, will also be studied.

CURRENT REPORTS AND PUBLICATIONS

(a) James P. Doll (1959). "Instability of Penicillin in Dubos Media." *Am. Rev. Resp. Dis.* 80, 262-263.

A STUDY OF THE INFLUENCE OF PASTEURELLA TULARENSIS
ON THE METABOLISM OF ANIMAL HOSTS

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ASSISTED BY Gennaro J. Miraglia and Margaret H. Stansberry

TASK NO. NR 103-088

CONTRACT Nonr-811(02)

OBJECTIVES

(a) To develop an investigation of the metabolism of tularemic animals with particular reference to disturbances in energy metabolism during infection, (b) to study in greater detail the immune response in tularemic rats with emphasis on the role of cellular defenses during infection and upon recovery from the disease.

ABSTRACT

Continued studies of the protective role of phosphorous compounds administered to tularemic rats were conducted. Creatine phosphate and KH_2PO_4 prolonged the lives and reduced the mortality rate of rats infected with P. tularensis. However, K_2HPO_4 was shown not to be protective. When the administration of KH_2PO_4 was delayed until 24 hours after infection no protective effect could be demonstrated.

Leucocytes harvested from the peritoneal cavity of rats recovered from tularemia were transferred to normal rats. These recipients exhibited a high degree of resistance to tularemic infection. The mortality rate among such animals was lower by 50 per cent than for infected control rats.

That this appears to be an immune response is suggested by the fact that leucocytes similarly harvested from normal rats do not confer any resistance or immunity upon recipient animals.

PLANS FOR FUTURE

(a) Further study of energy metabolism in tularemic animals with emphasis on the protective effect of phosphate administered during infection, b) continued investigation to elucidate the mechanism of this protective effect, including studies of tissue and serum ATP levels in infected rats, and in infected rats treated with phosphate, c) continued investigation of the cellular body defenses of tularemic rats.

COMPARATIVE BEHAVIOR OF RABBIT PERITONEAL MONOCYTES
INDUCED BY VARIOUS INFLAMMATORY AGENTS

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ASSISTED BY Patricia Schneider

TASK NO. NR 103-093

CONTRACT Nonr-222(72)

OBJECTIVES

(a) To study the responses of monocytes stimulated in the rabbit peritoneum by various irritants in order to determine the cause of discrepancies in pertinent literature on cellular immunity studied under conditions of cell culture.

ABSTRACT

A study has been completed of the responses of monocytes to the cytolytic action of mycobacterium tuberculosis, var. hominis, and Brucella melitensis. Monocytes were induced by inflaming the peritoneum with (a) "Klearol", a light mineral oil; (b) dextran, as used for human intravenous injection; (c) glycogen; (d) sodium caseinate; and (e) heat-killed B. melitensis. Monocytes were obtained from normal rabbits and from rabbits immunized against brucella infection by B. melitensis, strain Rev I, and against tuberculosis by the B.C.G. strain of M. tuberculosis. All procedures were otherwise as stated in earlier publications, where the Machaness method of observing monocytes has been described.

Monocytes induced by sodium caseinate, glycogen, and dextran behave in a manner completely opposite to those stimulated by mineral oil and killed brucella cells. The latter monocytes from normal animals are susceptible to the lytic action of the virulent bacilli even when suspended in serum from immune animals. Monocytes from immune animals are resistant to the lytic effect only when suspended in serum of animals sensitized to a wide variety of antigens, but are sensitive to the lytic action maintained in serum of normal animals. Monocytes induced by glycogen, dextran, or caseinate harvested from normal animals and maintained in normal serum resist the lytic action, whereas they are susceptible when taken from immune animals and maintained in serum of immune animals.

PLANS FOR FUTURE

To elucidate the metabolic events preceding the lytic stage, for both the intracellular parasite and the host cell.

CURRENT REPORTS AND PUBLICATIONS

Sanford S. Elberg and Patricia Schneider, 1960. Response of Monocytes in Relation to Nature of Inflammatory Irritant, in preparation.

FACTORS AFFECTING GERM TUBE DEVELOPMENT
OF PUCCINIA UREDOSPORES

Frederick G. Smith

ASSISTED BY B.R. Irvine

TASK NO. NR 103-109

CONTRACT Nonr-585(00)

OBJECTIVES

(a) To determine what factors control germination and germ tube differentiation of P. coronata uredospores, (b) to establish conditions permitting extended hyphal growth.

ABSTRACT

Further analysis of data on cation interaction in spore germination on gelatin has led to an improved formulation of the receptor site equilibrium expression. A search was made for suitable buffers to permit similar study of cation interaction on other substrates. Spore germination was more sensitive to buffer constituents on agar or water than on gelatin, but buffers consisting of lactate, tartrate, or malate and Tris appear to be satisfactory.

More extensive evidence of a volatile inhibitor of spore germination was observed by comparing germination of dense spore suspensions in open and closed vessels. However, experiments designed to demonstrate diffusion of the inhibitor across an air gap or into aqueous media on which spores were incubated were inconclusive. It appeared that the P. coronata inhibitor is either too unstable, too volatile, or is not sufficiently water soluble to be studied by the methods used. Experiments with chemicals reported to counteract self-inhibition in P. graminis tritici were more consistent. Coumarin increased germination in dense spore suspensions especially in closed vessels. It is clear, however, that the self-inhibition phenomenon is not as pronounced in P. coronata as in P. graminis tritici.

PLANS FOR FUTURE

(a) Study of the self-inhibition phenomenon in dense spore suspensions will be continued primarily to devise methods of overcoming it to facilitate metabolic work on spore germination. (b) If the present buffers prove suitable, study of the role of cations in germination and germ tube differentiation will be continued using ion exchange techniques, chelating agents, and surface enzyme inhibitors. (c) Methods for the study of spore and germ tube respiration and enzymatic capacity will be investigated.

CURRENT REPORTS AND PUBLICATIONS

F.G. Smith, H.M. Couey, and E.L. Sharp (1959), "Cation effects in the germination and germ tube development of Puccinia coronata." Proc. IX Intern. Bot. Cong. Vol. II p. 369.

BIOPHYSICAL INVESTIGATIONS ON
BACTERIOPHAGES

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ASSISTED BY James L. Allison, M.S., Carl E. Smith, M.S., and
David Goldstein, B.S.

TASK NO. NR 103-110

CONTRACT Nonr-624(03)

OBJECTIVES

(a) Refinement of the volume displacement method for determining the hydration of molecules in solution, (b) study of the birefringence resulting from the orientation of virus particles.

ABSTRACT

(a) Hydration - Our last annual report indicated that the use of organic indicator solutions, in contrast to sucrose solutions, gave hydration values for Escherichia coli independent of the density of the organic mixture employed. The method now has been refined and used to measure the hydration of Southern bean mosaic virus.

Since the absolute displacement is an inverse function of the cross-sectional area of the cell at the position of the interface through which the particles sediment, a new centerpiece for the synthetic boundary cell of the ultracentrifuge was designed and constructed for the purpose of minimizing this parameter. When this new centerpiece was employed with the synthetic boundary cell, in conjunction with o-dichlorobenzene-kerosene mixtures as the indicator solution, the hydration of Southern bean mosaic virus was found to be independent of the density of the bottom solution. For 9 determinations in a density range of 1.0179 to 1.1384 g/ml an average hydration of 0.98 ml/g of anhydrous Southern bean mosaic virus nucleoprotein was obtained, with the standard error of the mean being 0.05.

(b) Birefringence - In 1912 Wiener postulated that alignment of rods in a medium of different index of refraction will produce form birefringence, independent of whether the particles themselves are intrinsically birefringent. We are applying this principle to determine whether T2 bacteriophage can be oriented by physical forces such as centrifugal and electrical fields, and whether any resulting birefringence can offer information concerning the internal organization of the particle.

Material from a TMV pellet was streaked on a microscope slide and placed at 45° to the crossed Nicol prisms. When the slow direction of the compensator (Red I plate) was aligned parallel to the TMV streak,

the latter appeared blue. The blue color, indicative of positive birefringence, is that expected for rod-like particles aligned parallel to the axis of symmetry--in this case the direction of the streak. A similarly oriented streak from a pellet of T2 bacteriophage, however, demonstrated negative birefringence as revealed by an orange-red color. One may hypothesize that if, here too, the virus particles are oriented in the direction of the streak, the orange-red color may mean that the high percentage of nucleic acid (intrinsically negatively birefringent) of the bacteriophage is oriented within the virus particle and overwhelms the positive form birefringence. The nucleic acid strands would have to lie parallel to the long axis of the virus.

When drops of TMV and of T2 bacteriophage solution were placed on microscope slides, covered with coverslips and allowed to dry, birefringence was observed at the edges of the coverslips. Apparently, the surface tension forces oriented the virus particles parallel to the coverslip edge during drying. The TMV birefringence was positive; the T2 negative.

To test for possible orientation of T2 virus particles in an electric field, a microelectrophoresis cell was placed between the crossed Nicol prisms of a polarization microscope. In this manner birefringence was demonstrated. If this result is substantiated, it will mark the first time that the orientation of bacteriophage in an electric field has been observed.

PLANS FOR FUTURE

(a) To establish whether physical forces can induce orientation of virus particles, and determine whether any resulting birefringence may be interpreted on the basis of the particles' internal structure. (b) To carry out electron microscope studies on the small dissociation product (M.W. approximately 18,000) of TMV protein.

CURRENT REPORTS AND PUBLICATIONS

(a) Bendet, I. J., Allison, J. L., and Lauffer, M. A., "The Dual Sedimentation of T2 Bacteriophage-Discussion," Proceedings Fourth International Congress of Biochemistry 7, 199 (1958).

(b) Bendet, I. J., and Lauffer, M. A., "Virus Studies: Biophysical Methods," Medical Physics III (in press).

(c) Bendet, I. J., Smith, C. E., and Lauffer, M. A., "Hydrodynamic Volumes Determined by Immiscible Liquid Displacement," Arch. Biochem. Biophys. (in press).

STUDIES OF HOMOZYGOUS HAIRLESSNESS, SUPERFETATION AND PAPILLOMA IN MICE

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ASSISTED BY James Smith and Leone F. Barnes

TASK NO. NR 132-161

CONTRACT Nonr-1746(00)

OBJECTIVES

Rearing stocks of homozygous hairless mice for studies of the mechanism of hairlessness, its possible prevention, and various skin tests, including papilloma of the skin.

ABSTRACT

(a) Rearing of homozygous hairless deer mice, Peromyscus maniculatus gambeli. Out of the matings of 23 pairs of hairless P. m. gambeli males with hairless females (11 albino, nine colored and three mixed pairs), 13 were successful (five albino, five colored and three mixed pairs). An additional albino pair was found fertile; however, the mother cannibalized her offspring shortly after their birth. The remaining nine matings, consisting of five albino and four colored, were unsuccessful. The 13 fertile pairs had 24 pregnancies. They reared a total of 58 offspring to full maturity with an average of 2.4 individuals for each pregnancy. After birth all the young had hair which developed into a full coat, white or colored, depending on whether the parents were albino or colored, during the first two weeks of their lives; however, the following week the hair gradually fell out, usually first from the head and later from all over the body; within a month all the individuals became completely hairless. At the present time, the stock of colored hairless has reached the fifth and albino hairless the sixth generations, respectively. The offspring from these stocks when mated two to three months of age or older were found to be fertile and homozygous for hairlessness.

(b) Rearing of homozygous hairless house mice (Mus musculus). Out of 44 pairs of homozygous hairless M. musculus (16 albino and 28 colored pairs) nine were successful (five albino and four colored pairs). Two additional pairs gave birth to litters; however, they were cannibalized by the mothers the following day. Matings of the remaining 33 pairs were unsuccessful. A total of 37 offspring (18 albino and 19 colored) were reared by nine mothers to full maturity, an average of 3.7 individuals per pregnancy. Within a year the homozygous hairless stock of both colored and albino reached the fourth generation. All of the offspring had hair which developed normally to full coat within two weeks, after which the hair began gradually to disappear, and within a month all individuals, without exception, became hairless. The offspring from these stocks mated at two or three months of age or older were found fertile and homozygous for hairlessness.

(c) Superfetation or delayed fertilization in homozygous hairless mice. Out of 24 pregnancies from 13 pairs of Peromyscus maniculatus gambeli, superfetation or delayed fertilization was noted in three. From 10 successful pregnancies of homozygous hairless Mus musculus from nine pair, one was due to delayed fertilization.

(d) Spontaneous papilloma (warts) in hairless Mus musculus. In two instances, a spontaneous skin papilloma was noted in the stock of homozygous hairless Mus musculus. The papilloma was successfully transplanted into hairless mice. Studies are in progress to elucidate the etiology and other characteristics of this papilloma.

PLANS FOR FUTURE

Continuation of studies as described above.

CURRENT REPORTS AND PUBLICATIONS

(a) A. A. Packchianian and J. G. Sinclair (1959), "The rearing of homozygous hairless deer mice, Peromyscus maniculatus gambeli, with a note of superfetation or delayed fertilization." Texas Rep. Biol. and Med., 17, 229-236.

MECHANISMS OF VIRUS INFECTION: INTERACTION OF
GENETIC MATERIAL OF PHAGE AND BACTERIAL HOST CELL

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ASSISTED BY H. Rouhandeh

TASK NO. NR 103-172

CONTRACT Nonr-1170(00)

OBJECTIVES

(a) To vary the frequencies of stable transduction in Salmonella typhimurium by several means and to determine whether the frequencies of abortive transduction are altered to the same extent in each case, (b) to study in detail those conditions that produce increases in transduction frequency, (c) to study a mutant of S. typhimurium, designated as R3, which is capable of being transduced both stably and abortively, but which is resistant to phage infection (does not support multiplication), (d) to initiate research on the genetics of phage T3 of E.coli.

ABSTRACT

These studies have been concerned with an adenine-requiring mutant of Salmonella typhimurium strain LT 2.

Most of the results of experiments dealing with (a) and (b) above were described in the previous report and will be omitted here. Additional information has been obtained, however, which supports the hypothesis that, for either stable or abortive transduction, donor genetic material interacts in some way with the recipient genome.

(c) A mutant of S. typhimurium LT 2 was isolated (designated as R3), which was resistant to infection by phage P22. P22 was incapable of lysogenizing this strain, and it produced no plaques even when 10^9 phage were plated with R3 as the indicator host strain. P22 did not multiply in R3. However, phage P22 adsorbed to R3, and R3 could be used as a recipient for stable and abortive transduction of the adenine marker. There was no evidence of defective lysogenization. Phage P22 did kill about 10% of R3 cells, with no liberation of phage: no explanation could be found for this killing. Transductions could be carried out with virulent mutants of P22. This information supports the hypothesis that transducing phage are not confined to lysates prepared with temperate phage, nor is lysogenization involved in the process of transduction.

(d) Genetic variants of phage T3 were found that differed with regard to antigenic, host-range and morphological (electron microscope observation) properties. The relationship of these properties to each other was studied in detail while the senior investigator was at the Institute of Microbiology, University of Copenhagen, Denmark, during the past year.

PLANS FOR FUTURE

The contract with ONR was terminated in August, 1959. Future research will deal with genetics of phage T3.

CURRENT REPORTS AND PUBLICATIONS

(a) H. Rouhandeh (1959), "Comparison of stable and abortive transduction in an adenine-requiring mutant of Salmonella typhimurium." Ph.D. thesis.

(b) A. Eisenstark, O. Maaløe, and A. Birch-Andersen (1960), "Genetic variants of phage T3." Proceedings, 60th Annual Meeting of Society of American Bacteriologists, Philadelphia, May, 1960.

THE PHYSIOLOGY OF PLEUROPNEUMONIALIKE ORGANISMS
AND THEIR RELATION TO BACTERIA

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ASSISTED BY G. H. Rothblat and J. E. Boughton

TASK NO. NR NR 103-199

CONTRACT Nonr-551(31)

OBJECTIVES

(a) To study the function of the lipoprotein growth factor, (b) to elucidate the energy sources of PPLO and, (c) to study the relation of PPLO to bacteria.

ABSTRACT

Parallel chemical and radioisotope analyses of the sterol content of parasitic strains showed that 10 to 15 percent was the starting material, cholesterol, the remainder, non-saponifiable, non-digitonin precipitable lipid. At least 3 sterols are present. Sterol represents about 18 percent of the dry weight of the cells and about 30 to 40 percent of the dry weight of the cell membrane. Both resting and growing cells take up cholesterol-4-C¹⁴ by an irreversible adsorption process. Sterol can be removed only by solvent extraction. Uptake of cholesterol is a property common to and specific for PPLO. Bacteria adsorb insignificant amounts. Saprophytic PPLO which do not require the lipoprotein growth factor convert all the cholesterol to other non-saponifiable, non-digitonin precipitable lipid. They are also capable of de novo synthesis of a lipid of similar character.

Uptake of cholesterol is affected by cell concentration, time, temperature and pH as expected of an adsorption process. Respiratory, oxidative-phosphorylation inhibitors, heating of cells, the presence of other sterols and alteration of the lipoprotein or cells by protein end group reagents has little effect on sterol uptake.

A correlation was found to exist between the ability of a protein to support growth and its ability to regulate cholesterol uptake. Although all proteins capable of supporting growth can prevent growth inhibition or lysis induced by surface active compounds, some inactive proteins likewise possess this capability. A correlation exists between the ability of a surface active compound to support growth and its ability to facilitate the aqueous solubility of cholesterol.

PLANS FOR FUTURE

(a) Continue study of the function of the components of the lipoprotein growth factor, (b) attempt to define conditions for reliable experimentation on oxidative activity of "parasitic" PPLO, and (c) continue examination of the relation of PPLO to bacteria.

CURRENT REPORTS AND PUBLICATIONS

(a) P. F. Smith and G. Rothblat (1959), "Relation of PPLO to bacteria." Proc. N. Y. Acad. Sci. (in press).

(b) R. J. Lynn and P. F. Smith (1959), "Chemical composition of PPLO." Proc. N. Y. Acad. Sci. (in press).

(c) P. F. Smith (1959), "Nutritional requirements of PPLO and their relation to metabolic functions." Proc. N. Y. Acad. Sci. (in press).

STUDIES ON SPIROCHETOSIS AND TRYPANOSOMIASIS

A. Packchanian

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ASSISTED BY James Smith, Jack E. Stevens and Xavier Manrique

TASK NO. NR 103-200

CONTRACT Nonr-1061(00)

OBJECTIVES

Biologic, immunologic and chemotherapeutic studies of exotic diseases.

ABSTRACT

(a) A new serotype of Leptospira. From the blood of a patient (who resided in Galveston, Texas, for the last seven years), Leptospira was cultured in vitro successfully. This strain failed to agglutinate with antisera of the following serotypes: australis, ballum, bataviae, bovis (Israel), canicola, grippotyphosa, hyos, icterohemorrhagiae (AP Type I), pomona, pyrogenes, sejroe, and T-117 (hebdomadis). However, it agglutinated with antisera of a European strain of autumnalis in dilution of 1:1,000 and with the Fort Bragg, USA, strain (autumnalis) in a dilution of 1:100. The agglutination reaction was over 1:30,000 with the serum of the convalescent patient from which the strain was isolated. Studies are in progress to determine whether or not this is an entirely new serotype or identical to one of the known serotypes. Studies of the susceptibility and resistance of various species of rodents to this new strain are now in progress.

(b) Leptospira and Leptospirosis. A paper entitled "Differential diagnosis of infectious hepatitis, leptospirosis and yellow fever" was presented before the Second International Congress of Infectious Pathology at Milan, Italy, on May 6, 1959. The following represents the abstract of the communication. Three major epidemics and several small outbreaks of infectious viral hepatitis in the United States were described. The increased incidence of leptospirosis in man and animals in the United States was pointed out. Methods of diagnosis and differentiation of leptospirosis, infectious hepatitis and yellow fever from each other was outlined and discussed with special reference to epidemiology, histopathology, immunology, serology and animal inoculation tests.

(c) Chemotherapy of Borrelia anserina infection in chicks with newer antibiotics. Studies were completed pertaining to the chemotherapeutic effect of the following four antibiotics: sodium amphotycin, kanamycin sulfate, telomycin and tetracycline HCl. Each of these antibiotics in adequate dosage levels when administered intramuscularly was found curative. The chemotherapeutic index based on Maximum Tolerated Dose over Minimum Curative Dose (LD_{50}/CD_{100}) of these antibiotics against B. anserina

infection in chicks with 1 or 4 daily treatments was found to be approximately as follows: sodium amphotycin, 6.25-10; kanamycin sulfate, 4.5-7.5; telomycin, 2.75-6.25; and tetracycline HCl, 30. The chemotherapeutic index based on LD_{50}/CD_{50} of these compounds was in all cases higher than that expressed above.

(d) Chemotherapy of Trypanosoma gambiense infection in mice with new compounds. A total of 112 new chemicals and antibiotics were screened against Tr. gambiense infection in mice (Mus musculus). Of this number, two had a suppressive effect on the course of the disease, while four were curative with a low chemotherapeutic index.

(e) Studies on Endotrypanum schaudinni. Four sloths (Choloepus didactylus) received from Colombia, South America, were found naturally infected with E. schaudinni. The trypanosomes were found chiefly within the red blood corpuscles with no apparent movement, although a few parasites were extracellular and motile. This interesting parasite was cultured in vitro on several occasions. The animal inoculation test, method of transmission and biology of this unique trypanosome are now under investigation.

PLANS FOR FUTURE

Continuation of studies of the biology, immunology and chemotherapy of exotic diseases.

CURRENT REPORTS AND PUBLICATIONS

(a) A. Packchamian (1959), "On the cultivation of Trypanosoma brucei in vitro." Am. J. Trop. Med. & Hyg., 8:168-174.

(b) A. Packchamian (1959). "Differential diagnosis of infectious hepatitis, leptospirosis and yellow fever." 2nd Int. Cong. Infect. Path., Milan, Italy, May 6-10, 1959.

PROTEIN AND NUCLEIC ACIDS SYNTHESIS AND ENERGY-YIELDING PATHWAYS IN
SHIGELLA AND THE EFFECTS OF ANTIBIOTICS UPON THESE MECHANISMS

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ASSISTED BY Sylvia F. Pan and Robert B. Yee

TASK NO. NR 103-207

CONTRACT Nonr-2759(00)

OBJECTIVES

(a) To study the mechanisms of protein and nucleic acid synthesis of Shigella, (b) to compare these pathways in sensitive and antibiotic resistant strains of Shigella, and (c) to investigate the action of antibiotics on both synthesis and energy-yielding mechanisms.

ABSTRACT

The effect of low concentrations of chloramphenicol (0.5 - 2.0 ug/ml) on protein and nucleic acid synthesis by a strain of Shigella flexneri 3 growing in a mineral salt - glucose medium has been studied. Ammonium monobasic phosphate was used as the nitrogen source. It was found the antibiotic markedly depressed both protein synthesis and ammonia assimilation with a concomittant stimulation of ribonucleic acid (RNA) synthesis. However, the inhibition closely paralleled the retardation of cell multiplication. In order to determine whether the observed inhibition of protein synthesis and ammonia assimilation was the cause or the effect of decrease in growth, studies with washed cell suspensions were made. Very little growth in the synthetic medium during the incubation period employed in the experiments was detected. The results obtained demonstrated that in the presence of 0.5 - 2.0 ug chloramphenicol/ml protein synthesis was markedly inhibited and the net amount of RNA produced was increased. Much higher concentrations of the antibiotic were required for any measurable retardation of ammonia assimilation.

Of the amino acids tested, only L-aspartic acid could be substituted for the ammonium salt to yield comparable or better growth in synthetic media. This amino acid was therefore employed in experiments with washed cell suspensions. In these experiments, as in ones using the ammonium salt, low concentrations of chloramphenicol markedly inhibited protein synthesis and stimulated RNA synthesis. A study is in progress to determine if aspartic acid assimilation and the metabolism of the amino acid are sensitive to low concentrations of the drug. Various investigators have reported that glycine, tyrosine and phenylalanine may reverse chloramphenicol inhibition. Experiments are being carried out to ascertain the effect of these amino acids on the observed depression of protein synthesis in S. flexneri 3.

The amino acid content of hydrolysates of cell free extracts and whole cells was determined by column chromatography. Seventeen ninhydrin

positive peaks were observed. Glutamic and aspartic acids, alanine, glycine and leucine were the most abundant amino acids obtained. The specific amino acid/leucine ratio varied with the nitrogen source supplied to the bacteria. Cells grown in tryptose phosphate broth gave the highest ratio for most of the amino acids. Cells grown in synthetic medium containing aspartic acid as the nitrogen source had a lower ratio while cells obtained in the presence of ammonium phosphate had the lowest values. Although the total Kjeldahl nitrogen levels were comparable the amino acid content of hydrolysates of cells grown in medium containing low concentration of chloramphenicol (0.5 ug/ml) was lower than that of hydrolysate of cells grown in antibiotic-free medium. The amino acid composition of chloramphenicol resistant cells is being determined.

CAUSES OF VARIATION IN THE RUST DISEASE SYNDROME

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ASSISTED BY R. G. Hacker

TASK NO. NR 103-216

CONTRACT N9 onr 824(02)
Amendment No. 8

OBJECTIVES

(a) To determine the influence of inoculum density on the type of infection caused by Puccinia graminis var. tritici as affected by certain environmental factors such as light, nutrition, and temperature, (b) to determine the effects of antagonism or cross protection among races of rust fungi in relation to pathogenicity and symptom expression, as affected by light, nutrition, and temperature.

ABSTRACT

Uredospores of several rust fungi have been mass-produced in the greenhouse. Preliminary to mass production of spores, each rust organism was increased from single-pustule isolates to insure purity.

In order to determine uniformity of spore fall in the settling tower, slides were placed on the floor of the tower, and random counts of 25 microscope fields were taken within the center area of each slide. The uniformity of spore fall was reflected in the lack of any appreciable variability among replications in inoculation of seedlings as determined by pustule counts and distribution.

I. Influence of nutrient deficiencies on pustule development.

Seedlings were grown in vermiculite until the endosperm food reserves were exhausted, then washed and transplanted into volcanic ash-filled capsules, which were then placed in paraffin-lined sheet metal trays and flooded as required with liquid nutrient solutions in which the N, P, and K levels were varied.

When the seedlings became established, selected leaves were held flat by means of masking tape and were inoculated. Later, pustule counts were taken on 5 centimeter middle segments of leaf surface.

Low levels of nitrogen, phosphorus, and potassium in the nutrient media produced varying degrees of chlorosis and stunting of the seedlings. The effects were most severe for low levels of nitrogen and potassium and only slightly evident in the phosphorus-deficient plants. Plants grown in a complete nutrient medium were normally green and vigorous.

Severely chlorotic plants were not infected by the rust fungi, whether the deficiency was nitrogen, phosphorus, or potassium. Mild and moderate chlorosis reduced pustule development correspondingly. As the degree of chlorosis increased, the pustule counts decreased, and uredia were more weakly developed. "Resistant" type pustules having wide chlorotic borders were typical of the plants in these categories. Both parasite and host seem to have similar requirements for nitrogen, phosphorus, and potassium.

II. Tests of antagonism and cross protection between races and species of rust fungi.

Using a split plate technique, uredospores were seeded onto agar in several combinations. There were no evidences of inhibition of spore germination between any of these fungi. Germination counts were uniformly the same in the zones of contact as in distal areas. Likewise, germ tubes were freely intermingled at the zone of contact.

Tests of cross-inoculation with different rust species were made on Onas wheat, using uredospores of P. graminis var. secalis and P. coronata as primary inocula, followed in 48 hours with P. graminis var. tritici, R 56. The prior presence of the non-pathogen did not interfere with the normal development of the pathogenic species. It is inferred that there is no interference of inhibition in these combinations. Numerous other combinations are being investigated.

Collections of uredospores of yellow stripe rust were made at the University of Wyoming Agronomy Farm, Laramie, Wyoming. Onas wheat was so severely infected with this rust that it was possible to collect several test tubes of pure spores, using the electrostatic precipitation method. Quite inadvertently, the authors noticed that the tubes of spores gave off a pleasant, fruity aroma similar to that of fresh tangerines. Speculating that the odor possibly is due to the presence of terpins, tests are being made to see whether these might be inhibitors.

PLANS FOR FUTURE

(a) To test further the effects of nutrient levels on rust development (b) to test other combinations of rust fungi for possible antagonism or cross protection between species or races.

CURRENT REPORTS AND PUBLICATIONS

(a) L. J. Petersen. 1959. Relations between inoculum density and infection of wheat by uredospores of Puccinia graminis var. tritici. Phytopathology, 49, 607-614.

(b) L. J. Petersen. 1958. "Studies on the relationship between inoculum density and infection of wheat by uredospores of Puccinia graminis var. tritici." M. S. thesis, Colorado State University. Technical Report Task Order NR 103-216; Contract N9 onr 824(02).

(c) L. J. Petersen. 1956. "A method for observing stomatal penetration by uredospore germ tubes of Puccinia graminis f. sp. tritici." Phytopathology, 46, 581-582.

(d) A. R. Weinhold. 1955. "Rate of fall of urediospores of Puccinia graminis tritici Erikss. and Henn. as affected by humidity and temperature." M. S. thesis, Colorado State University. Technical Report Task Order 82402; Contract N9 onr 82400.

MEASUREMENT OF SMALL ABSORBANCY CHANGES IN MICROSPPECTROSCOPY
AND OF SMALL INTENSITIES IN MICROFLORIMETRY

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University of Pennsylvania

ASSISTED BY Dr. B. Thorell, Miss G. Cullen, Messrs. Cowle, Bicking, Kravitz

TASK NO. NR 103-242

CONTRACT Nonr 551-26

OBJECTIVES

To apply microspectrophotometry and microflorimetry to various biological substances and to develop techniques for microspectrophotometry at liquid temperatures of liquid nitrogen.

ABSTRACT

The microflorimeter has been studied intensively in the past year and the development of a method of obtaining oxygenation and disoxygenation of biological material without causing their movement under the coverslip has been considered. The use of a chlorella yeast mixture seems appropriate wherein the ratio of chlorella to yeast cells is chosen to give adequate oxygenation under illumination which will persist for five minutes in the dark.

The microspectrophotometer has been adapted for measurement of the α bands of cytochromes in cells and liquid nitrogen temperatures. An intensive series of experiments have been carried out in collaboration with Dr. Bo Thorell and the work is in the process of preparation.

CURRENT REPORTS AND PUBLICATIONS

- (a) R.P. Perry, B. Thorell, L. Akerman, and B. Chance (1959). "Localization and assay of respiratory enzymes in single living cells. Absorbancy measurements on the Nebenkern." *Nature*, 184, 929-931
- (b) B. Chance and B. Thorell (1959). "Localization and assay of respiratory enzymes in single living cells. Fluorescence measurements of mitochondrial pyridine nucleotide in aerobiosis and anaerobiosis." *Nature*, 184, 931-934
- (c) B. Thorell and B. Chance (1959). "Localization and assay of respiratory enzymes in single living cells. Absorbancy measurements on liver and kidney cells." *Nature*, 184, 934-935
- (d) B. Chance and V. Legallais (1959). "Differential microfluorimeter for the localization of reduced pyridine nucleotide in living cells." *Rev. Sci. Instr.*, 30, 732-735
- (e) B. Chance, R. Perry, L. Akerman, and B. Thorell (1959). "Highly sensitive recording microspectrophotometer." *Rev. Sci. Instr.*, 30, 735-741
- (f) B. Chance and B. Thorell (1959). "Localization and kinetics of reduced pyridine nucleotide in living cells by microfluorometry." *J. Biol. Chem.*, 234, 3044-3050

- (g) B. Thorell and B. Chance (1959). "Microspectrography of respiratory enzymes within the single mammalian cell under different metabolic conditions." *Exptl. Cell Research*, 18, No.4
- (h) R. Perry, B. Thorell, L. Akerman, and B. Chance (1960). "Microspectrophotometric measurements of the cytochromes in a single in vivo mitochondrion. *Biochim. et Biophys. Acta*, in press.

ENZYMATIC FACTORS CONCERNED WITH THE VIRULENCE OF
CERTAIN BACTERIAL SPECIES

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ASSISTED BY W. J. Begue, M. Freundlich and J. R. Waller

TASK NO. NR 103-250

CONTRACT Nonr-1514(00)

OBJECTIVES

To study factors, particularly nutritional, which influence the enzymic activity and/or constitution of bacterial cells.

ABSTRACT

Increased nutritional requirements of *Saccharomyces cerevisiae* as a result of incubation at 38 C. It is known that environment exerts a marked influence on the nutritional requirements of microorganisms. For example, changes in the temperature of incubation may alter the nutritional demands of microorganisms and in most instances where this phenomenon has been demonstrated an increase in temperature is accompanied by a gain in nutritional requirement. During studies on the nutrition of *S. cerevisiae* it was observed that several strains were unable to initiate growth or grew very poorly at 38 C in a chemically defined medium adequate for optimum growth at 30 C. In contrast, these organisms proliferated equally well at both temperatures after inoculation into a complex medium. Furthermore, the addition of yeast extract to the synthetic medium resulted in growth of the organisms at 38 C. Subsequent investigation revealed that the active component of yeast extract was replaceable by calcium pantothenate. Addition of this vitamin to the defined medium supported growth of the yeasts at 38 C to the extent of 40 to 82 per cent (average 61 per cent) of that obtained at 30 C in the same medium containing beta-alanine instead of pantothenic acid. Further study showed that sodium pantoate or its lactone replaced the effect of pantothenate but in concentrations approximately 1,000 times greater than the amount of pantothenate required. It appears that the synthesis of pantothenate accomplished by these organisms at 30 C is prevented by incubation at the elevated temperature.

Accumulation of biotin by *Lactobacillus arabinosus*. Factors affecting the uptake of biotin by resting cell suspensions of *L. arabinosus* (17-5) are under investigation. In contrast to earlier studies employing biotin deficient cells, the present work involves biotin sufficient cells. Such cells were harvested from Skeggs-Wright medium after 48 hours growth at 30 C, washed three times with saline and suspended in phosphate buffer (pH 6.8) in the presence of biotin at 37 C. The reaction was stopped by boiling, the cells recovered by centrifugation and the washed cells treated with 6 N H₂SO₄ at 121 C for 60 minutes to

release bound biotin which was assayed microbiologically with L. arabinosus. The specific factors studied were the influence of time, the effect of cell and substrate concentration and the pH and temperature optima. A linear relationship was obtained when biotin accumulation was plotted against time with completion occurring within 90 minutes. The rate of biotin uptake was found to be directly proportional to the concentration of cells. A straight line relationship was obtained when the reaction velocity was plotted against the substrate concentration except that at high extracellular biotin concentrations the curve fell off abruptly. The pH optimum was found to be 6.8 with activity falling off quite rapidly on either side of the optimum. Temperature studies revealed an optimum at 37 C with a Q_{10} between 30 and 37 C of 1.8. The participation of an enzyme system in the accumulation of biotin by cells of L. arabinosus is inferred from these results.

PLANS FOR FUTURE

To continue investigation of the problems cited above as well as a study of the effects of glucose on the tryptophanase and tryptophan synthetase system of E. coli.

CURRENT REPORTS AND PUBLICATIONS

(a) L. G. Jayko and H. C. Lichstein (1959), "Nutritional factors concerned with growth and lecithinase production by Clostridium perfringens." J. Infect. Dis., 104, 142-151.

(b) W. J. Begue and H. C. Lichstein (1959), "Increased nutritional requirements of Saccharomyces cerevisiae as a result of incubation at 38 C." Bacteriol. Proc., p. 113.

A STUDY OF THE PROPERTIES AND KINETICS OF HEAT RESISTANT
ENZYMES FOUND IN BACTERIAL SPORES

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ASSISTED BY G.G. Krishna Murty, H.M. Nakata, I.D. Goldberg, & F.E. Engel

TASK NO. NR 103-251

CONTRACT ONR-1834(09)

OBJECTIVES

(a) To study the biochemical changes occurring during sporulation of aerobes and anaerobes and (b) to search for enzymes in anaerobic spores and study their heat resistance.

ABSTRACT

The vegetative cells of B. cereus T do not seem to possess a completely oxidative pathway for the breakdown of glucose. During growth, the cells catabolize glucose to intermediate acids which accumulate in the medium causing a lowering of the pH. With the onset of sporulation, these acid intermediates are metabolized with a concomitant rise in the pH of the culture.

Among the pyridine carboxylic acids tested, only alpha-picolinic acid specifically inhibited sporulation under specified conditions, the others having no effect. In such cultures inhibited from sporulation, the pH stayed at the lowest level reached during vegetative growth indicating that alpha-picolinic acid interfered with the further metabolism of the acid intermediates. Alpha-picolinic acid is a powerful chelating agent and analogue of nicotinic acid. That the inhibitory effect may be due to chelation of some metal and not due to competition with nicotinic acid is evidenced by the fact that the inhibition could be overcome by Zn, Co or Ni but not by nicotinic acid.

The acids accumulating in a normally growing and sporulating culture could be extracted and shown to reverse the inhibition by alpha-picolinic acid. These acids were identified to be pyruvic and acetic and the amount of these was shown to vary in accordance with changes in the pH of the culture. The inhibition of alpha-picolinic acid could also be overcome by yeast extract but not by the ash from yeast extract. The other extractable acids were shown to be responsible for the effects of yeast extract. This metabolic lead was followed up by testing a number of amino acids, sugar acids and other organic acids for their ability to reverse the inhibition of alpha-picolinic acid. Of all these compounds tested, only aspartic acid and asparagine among the amino acids, and pyruvic, acetic, citric, cis aconitic, isocitric, succinic, malic, oxalacetic, malonic, propionic, methyl malonic and formic among the organic acids reversed the inhibition. With the exception of formate, all the others could be fitted into the glyoxylic acid cycle suggesting that the acid intermediates may be metabolized, at least partly, through this

cycle and that succinic acid may be the immediate precursor of DPA. Experiments with bisulfite and fluoroacetic acid lend further support to the above assumption.

According to the above scheme of events, pyruvic and succinic acids will be key intermediates in the metabolism of a sporulating cell. If the carboxyl groups of these acids are involved in the synthetic processes of such a cell, one would expect the esters of these acids to be inhibitory. In fact, ethyl pyruvate, diethyl succinate and diethyl malonate have been found to inhibit sporulation. The last two specifically inhibit sporulation while ethyl pyruvate also inhibits germination. The effects of these esters and the enzymes involved are under study.

PLANS FOR FUTURE

(a) To study the nature of the enzyme system involved in sporogenesis, (b) to study the fate of the acids accumulating during vegetative growth, and (c) to study the effect of ethyl pyruvate on germination.

CURRENT REPORTS AND PUBLICATIONS

(a) Halvorson, H. O., (1959), Dormancy, Germination, and Outgrowth. *Bacteriol. Rev.* 23, 267-272.

(b) Nakata, H. M. and Krishna Murty, G. G. (1959), Biochemical changes occurring during the transition from vegetative growth to sporulation in Bacillus cereus T. *Bacteriol. Proc.*, p. 39.

(c) Krishna Murty, G. G. and Halvorson, H. O. (1959), Biochemical changes occurring during sporulation of Bacillus cereus T. I. Inhibition of sporulation by alpha-picolinic acid. In press.

(d) Krishna Murty, G. G. and Halvorson, H. O. (1959), The role of the glyoxylic acid cycle with sporulation of Bacillus cereus T. *Bacteriol. Proc.*, p. 39.

NATURE OF COLICINES AND THEIR FUNCTION IN THE
ECOLOGY OF THE FECAL FLORA IN HEALTH AND DISEASE

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ASSISTED BY Messrs. Bourgeois, Minken, Prescott, Brubaker, Nemeth

TASK NO. NR 103-260

CONTRACT Nonr-761(02)

OBJECTIVES

(a) To study the antagonistic properties of coliform and other intestinal bacteria against Shigella and other pathogenic enteric bacteria and viruses, (b) To study the colicine sensitivity of related intestinal organisms, (c) to attempt to reconcile certain discrepancies brought out with different technics, (d) to attempt the isolation of colicine, (e) to study the role of colicines in the natural ecology and work out possibilities for their practical use.

ABSTRACT

Colicine sensitivities have been tested with strains of Shigella dysenteriae, Sh. boydii and Sh. flexneri to determine any relationship between such sensitivities and species serotype. One or more strains of each of the recognized and provisional serotypes of these three species were tested, 288 in all. From 24 to 28 different colicine-producing cultures were used, one study utilizing 24, a second 26, and the third 28. The "chloroform killed" method was used, although in most cases correlation with the "living" method was attempted. We feel the killed method more nearly represents the action of colicine than the living method in which other factors than colicine may operate. Previously reported work revealed that for strains of Vibrio, some of which were inhibited by colicines, such action only resulted when both the Vibrio and the coliform "producer" were growing concurrently in contact. This contrasts with coliform colicine action on strains of Corynebacterium which only took place when the "dead culture" method was used. With rare exceptions colicine action within the Enterobacteriaceae takes place by both methods. An attempt was made to substitute 20% ethylene oxide for chloroform as a killing agent. Delays in obtaining the Ben Venue sterilizer needed prevented completion of the experiment.

The results of tests involving 288 organisms, each tested against from 24 to 28 "producers" gives more than 7500 data which unfortunately do not fall into very clear cut patterns. In general the species Shigella boydii is more resistant to colicines than the other two species. Of the 169 strains of boydii tested 19.5 were completely resistant to all 28 colicines used. It was also found that significant sensitivity differences occurred between smooth (S), rough (R), and mucoid (M) variants. By contrast all 85 strains of Sh. dysenteriae were susceptible to one or more of the 24 colicines used and of 34 strains of Sh. flexneri tested against 26 colicines only strain 6-1 of serotype 6 was resistant to all colicines used in that study. Colicines differ greatly in effectiveness. Coliform CA23 producing colicine "D" inhibited 203 Shigella strains, K235 producing colicine "K" 152 strains and a Sh. sonnei producer from

which comes "S3" 93. By contrast some colicines such as "A", "S4", and "F1" inhibited few Shigella strains. Even though no clear cut pattern of reactivity is seen in this study of only 288 strains there are several suggestive leads which will be presented in a final report. It is clear that bacteria producing colicines do inhibit dysentery bacilli to a significant degree in vitro. Whether or not inhibition occurs in the bowel is another question. It may also be significant that fewer of the Sh. boydii strains are inhibited by our rather complete battery of colicines than is the case for Sh. flexneri or Sh. dysenteriae. In an earlier study on susceptibility patterns of Salmonella 77 strains were tested against 40 colicine producers. Of their 93% were susceptible to one or more inhibitors. "Patterns" of susceptibility were quite different from those of Shigella strains. The most active colicines were "534", "J", "S3", "E" and "V". "K" inhibited only 1 of the 77 strains and "D" only 21, although "S3" was active against strains from both genera.

An attempt was made to prepare and purify colicine "534" by ion-exchange chromatography following growth of the culture on agar instead of on broth followed by extraction of the colicine from the washed agar. The first two "runs" in the diethylamino cellulose columns gave increasingly good yields, followed on third attempt by complete loss of activity. Time did not allow further pursuit of this lead. The loss of activity during chromatography was observed in earlier work and may be significant.

During the summer a "pilot" study on the effect of colicines on enteroviruses was carried out with colicines "534" and "V", as pure as we could produce them, and possessing demonstrated activity. We tested the activity of these colicines on tissue culture material and on five viruses growing on it. Colicine had no effect on growing monkey kidney cell lines or on "Hep II" cells. Nor did it affect the growth on these mediums of five viruses tested: poliomyelitis type II, Coxsackie A-9, Coxsackie B, Echo 9 and Echo 4.

PLANS FOR FUTURE

The retirement of the senior investigator and the preoccupation of the junior responsible investigator makes it unlikely that all obvious plans for the future can be realized. We hope, however, (a) to check the work with colicines and viruses which we regard as an important point, (b) to look into the differences we have observed between the colicine action of a living "producer" and a killed culture, (c) to attempt again to recover colicine from the agar or broth in which it occurs when a producer culture is grown and attempt its purification, (d) to put together the work done in this laboratory on colicine dating back to our first studies with it begun in 1949 and produce some sort of a final report of possible usefulness to others.

CURRENT REPORTS AND PUBLICATIONS

None during the year.

THE NATURE OF RESISTANCE AND SUSCEPTIBILITY
TO INFECTIOUS AGENTS

E. D. Garber
University of Chicago

ASSISTED BY Susan G. Weinshelbaum

TASK NO. NR 103-267

CONTRACT Nonr-2121(14)

OBJECTIVES

(a) To survey the spectrum of biochemical mutants in toxigenic species of Pseudomonas after UV-irradiation, (b) to characterize the toxin from each species, (c) to determine the nature of resistance to wildfire disease of tobacco, (d) to correlate the presence of specific enzymes with pathogenicity in soft-rot bacteria, and (e) to establish the role of the nutritional status of the host with respect to resistance and susceptibility.

ABSTRACT

Toxigenic species of Pseudomonas may produce different spectra of biochemical mutants after exposure to UV and screening with penicillin. In certain species, approximately 85% of the mutants have the same or related nutritional requirements. In Ps. tabaci, the predominant mutant needs methionine, in Ps. phaseolicola and in Ps. coronafaciens, glutamic acid, aspartic acid, or proline, and in Ps. tomato, aromatic amino acids (including shikimic acid) or nicotinic acid. Other species have not yet demonstrated a predominant mutant type.

The methionine-requiring mutants of Ps. tabaci, Ps. glycinea, and Ps. phaseolicola grow in minimal medium plus dimethyl propiothetin. This compound presumably serves as a methyl donor in the conversion of homocysteine to methionine. If demonstrated to be valid, this would be the first case of an exogenous methyl donor for transmethylation in microorganisms. The available evidence suggests that methionine biosynthesis in Ps. tabaci does not involve cysteine and that the production of the exotoxin, a methionine antimetabolite, may be related to an unusual biosynthesis or utilization of methionine. In a closely related, non-toxigenic species, Ps. angulata, methionine biosynthesis involves cysteine.

Liquid cultures of Ps. tabaci grow in the presence of allylglycine and comparable cultures of Ps. angulata are inhibited. Since this compound is an antimetabolite for cysteine, it may be assumed that this amino acid may not occur in the free state in the former species. This observation conforms with other information on methionine biosynthesis.

Wildfire is a disease of tobacco incited by Ps. tabaci. Expressed sap from leaves of a resistant variety had pH 5.4 and from a susceptible variety, pH 5.9. The viable count in number of cells per ml of leaf homogenate taken from the site of inoculation was determined over a period of 15 days. In susceptible leaves, the viable count increased from 10^2 to 10^6 - 10^7 within 3 days and maintained these values for 12 days; in resis-

tant leaves, the viable count increased from 10^5 to 10^6 - 10^7 within 3 days and then rapidly decreased until no cells could be detected within 9-12 days. Resistance to this pathogen may be related to localized pH changes at the site of inoculation caused by the proliferating bacteria and the absence of a mechanism for the rapid diffusion of compound(s) responsible for the pH change or to a poor buffering of the acidic compounds produced by the bacteria.

Two pilot projects on a phytopathogenic fungus were completed. One project established that a pattern of virulence and avirulence occurs when biochemical mutants of Fusarium oxysporum f. pisi are inoculated into a series of susceptible varieties of the host. This observation demonstrated that the results of earlier work with pathogenic bacteria may be extended to pathogenic fungi. The second project involved the parasexual cycle, a phenomenon permitting genetic analyses in fungi without resorting to the sexual stage. It was shown that strong selective forces may operate to prevent the incorporation of certain recombinant nuclei into spores, thereby seriously interfering with the genetic analysis. Since fungal incitants of human and animal diseases will eventually be studied by biochemical-genetic methods, these observations may provide a basis for the design of future experiments.

PLANS FOR FUTURE

(a) To determine if the exotoxins of the toxigenic species of Pseudomonas act as antimetabolites for the same amino acid, (b) to determine if these exotoxins represent different analogs, (c) to relate the spectra of biochemical mutants with exotoxin from each species, (d) to study the mechanism in tobacco conferring resistance to Ps. tabaci, and (e) to isolate non-pathogenic mutants of soft-rot bacteria in an effort to determine the role of enzymes in pathogenicity.

CURRENT REPORTS AND PUBLICATIONS

(a) E. D. Garber (1959), "Further observations on biochemical mutants of Pseudomonas tabaci." Bot. Gaz., 120, 157-161.

(b) E. D. Garber (1959), "Biochemical mutants of toxigenic phytopathogens." Proc. IX Internat. Bot. Congr., 2, 130 (abstr.).

(c) E. D. Garber (1959), "Nutritional aspects of the host-parasite relationship." Proc. IX Internat. Bot. Congr., 2, 130 (abstr.).

(d) E. D. Garber and H. E. Heggstad (1958), "Observations on the pathogenicity of biochemical mutants of Pseudomonas tabaci." Phytopath., 48, 535-537.

(e) R. W. Tuveson and E. D. Garber (1960), "Genetics of phytopathogenic fungi. I. Virulence of biochemical mutants of Fusarium oxysporum f. pisi (Linf.) Snyder and Hansen." Bot. Gaz. (in press).

(f) R. W. Tuveson and E. D. Garber (1960), "Genetics of phytopathogenic fungi. II. The parasexual cycle in Fusarium oxysporum f. pisi." Bot. Gaz. (in press).

ENZYMES AND THE PATHOGENESIS OF TUBERCULOSIS

Max B. Lurie and Arthur M. Dannenberg, Jr.
University of Pennsylvania

ASSISTED BY W. E. Bennett, J. Wildhack and P. C. Walter

TASK NO. NR 103-271

CONTRACT Nonr-551 (24)

OBJECTIVES

To determine the role of various enzymes (a) in the destruction of tubercle bacilli and their toxic products, (b) in the mechanism of delayed hypersensitivity, and (c) in the liquefaction of caseous tissue. The effect of these enzymes on the pathogenesis of tuberculosis is to be investigated at three levels: (1) that of the cells, specifically enzymes of mononuclear exudate cells, (2) that of the tissues, specifically enzyme demonstration by histochemical techniques, and (3) that of the intact animal, specifically the inhibition or stimulation of its enzymes by the administration of various compounds.

ABSTRACT

(1) The tentative conclusion of last year's Progress Report was that tuberculous rabbit mononuclear exudate cells showed about a 20% non-specific increase in certain proteinase and esterase activities with respect to control rabbits. The results were on the borderline of statistical significance, and, unfortunately, were not confirmed by a repeat experiment on 42 animals. This second experiment did, however, suggest that the activity of a true lipase, hydrolyzing coconut oil, was elevated in mononuclears from the tuberculous group. A third experiment of 34 rabbits tended to confirm this impression. Its activity averaged 75% higher in the tuberculous group, and this was statistically significant. However, in spite of statistics, these results were not consistent. Two of the 17 pairs dropped, 6 remained unchanged, and 9 increased an average of 230%. We can only conclude that this is the most promising enzyme we have studied so far, and that specific lipids from the tubercle bacillus should be tested as substrates.

(2) In collaboration with Dr. Marvin S. Burstone of the National Institutes of Health, a histochemical study of mononuclear and polymorphonuclear exudate cells was begun. In addition to screening these cells for various enzymes, quantitative studies were made to determine what changes, if any, were occasioned in these cells by the in vitro phagocytosis of yeast organisms. The beauty of this method, which employs Mylar strips to hold the cells and immune serum to aid phagocytosis, is that the ingested particle and a specific enzyme are visually and simultaneously demonstrated in each phagocyte. Thus, its phagocytic activity and enzymatic activity can be quantitatively correlated by counting the number of particles ingested and the number of granules produced histochemically in its cytoplasm. To date, no correlation has been found. However, only the ingestion of yeast, and esterase, acid phosphatase and succinic dehydrogenase have, as yet, been tested. Although other enzymes and ingested particles may yield different results, it is also possible that a phagocyte's ingestive and digestive activities vary independently.

PLANS FOR FUTURE

(1) Biochemical studies: (a) To complete the comparison of enzymes of tuberculous and normal mononuclear exudate cells by including DNases, RNases, hyaluronidase and lysozyme; (b) to complete the comparison of the proteinases, esterases, lipases and nucleases of mononuclear and polymorphonuclear exudates; (c) to quantitatively assay these enzymes in mononuclears from rabbits under the influence of cortisone and also triiodothyronine.

(2) Histochemical studies: (a) To continue the correlation of ingestive and digestive functions of mononuclears and polymorphonuclears; (b) to study histochemically the pathogenesis of pulmonary lesions produced in rabbits by the inhalation of tubercle bacilli.

IN VIVO ACTION OF COMPLEMENT

A. P. McKee and W. S. Jeter
State University of Iowa

ASSISTED BY Solveig Ordell

TASK NO. NR 134-276

CONTRACT Nonr-1509(01)

OBJECTIVES

Our progress has been chiefly along three lines. (1) Ruling out spurious hemagglutinin as an influence on the anticomplement when injected in the body (2) Demonstrating the influence of anticomplement on resistance and immunity (3) We have a considerable quantity of data regarding the effect of anticomplement on blood cells but have not analyzed it adequately as yet.

ABSTRACT

Hemagglutination

In building antibody against a specific complement, (for reasons not clear to us), some antibodies are developed against red blood cells. We were able to show that this is not the cause of the anticomplement function, but rather is a spurious counterpart. We were particularly concerned about this especially because we thought this might reduce the anticomplement titer when it was put in vivo and had to come in contact with so many red blood cells.

Effect of Anticomplement on Resistance and Immunity

Mice were used as the test animal in the studies on resistance and immunity. An antibody prepared against mouse complement was used to overcome the mouse complement in vivo. Mice, unlike guinea pigs, do not become seriously ill following administration of enough anticomplement to bind most of their complement.

Staphylococcus aureus, Streptococcus pyogenes, Salmonella typhimurium, Clostridium perfringens, Pasteurella hemolytica, Pasteurella multocida, Diplococcus pneumonia, type A and A" influenza viruses have all been studied to some extent in regard to their ability to produce infection. Parallel groups of mice were used in each experiment. One group received normal rabbit serum, the other group was given rabbit antimouse complement. Infectivity titers and plate counts (viruses excepted) were used to properly record the challenge doses of the infecting agents. Single and multiple doses of anticomplement were given.

Results of these studies indicate the following:

1. Anticomplement reduces resistance from 0-90% depending on the infecting agent.

2. Anticomplement compromises immunity from 0-50% depending on the infecting agent.
3. A given series of experiments with one strain of organism gives reproducible and quite consistent results.
4. Complement appears to be very important when some infectious agents are used and appears to play little or no role when others are used.
5. It seems doubtful that complement plays the only important role in any of the infections studied.

Effect of Anticomplement on Blood Cells

We have had a considerable amount of irregular results where anticomplement effect on white blood cells was concerned. At times we find that anticomplement apparently effects in a deleterious fashion a high percent of white cells. More commonly than not we have found these to be the neutrophils, although we have also found that platelets may be effected adversely with anticomplement. The results are not always consistent and apparently we have an extra factor involved to explain some of this inconsistency. One of these extra factors, if there be more than one, could well be this hemagglutinin which we have discovered. We are now in the process of commencing the effect of anticomplement against the blood cells of animals after the hemagglutinin has been adsorbed. Perhaps this will iron out some of the irregularities and allow us to come to a reasonable conclusion in regard to the effect of anticomplement on white cells.

PLANS FOR FUTURE CURRENT REPORTS AND PUBLICATIONS

We expect to round out and conclude our studies on resistance and immunity within the next few months. The results of these studies will be (a) published (b) given as part of a symposium at the 1960 National Society of American Bacteriologists meeting in Philadelphia.

We hope to continue our temporarily shelved studies on white cells, source of complement and perhaps to reopen studies on hypersensitivity.

AMINO ACID METABOLISM IN BACTERIA

J. M. Prescott

Texas Agricultural Experiment Station

ASSISTED BY Ruth J. Hurley and W. T. Williams

TASK NO. NR 103-283

CONTRACT Nonr-2204(00)

OBJECTIVES

To study (in certain Streptococci): inhibition of bacterial growth by amino acids, (b) biological synthesis of amino acids, and (c) factors which influence requirements for nitrogenous compounds.

ABSTRACT

Acetate exerts a marked stimulation on the growth of Streptococcus bovis when this organism is grown in a medium containing ammonium sulfate as the nitrogen source (Progress Report, January, 1959). Further tests have shown that the presence of 15 mg. of sodium acetate per 6 ml. culture also improves slightly the growth obtained when several individual amino acids constitute the sole nitrogen source. The stimulation with amino acids is not so marked as that shown in the ammonium medium, however.

Clear-cut evidence for the participation of acetate in the synthetic mechanism of S. bovis was obtained by use of C^{14} labeled acetate as a tracer. Cells were grown in ammonium sulfate medium which contained sodium acetate, harvested while in the logarithmic growth phase, then immediately resuspended in medium containing carboxyl-labeled acetate, and allowed to grow for one hour. The cells were then collected, washed, dried, and counted. A rapid and heavy uptake of the C^{14} labeled acetate was found. The cells were fractionated by extraction with boiling water to remove free amino acids, and both the extract and the insoluble residue were counted. The extract contained much less radioactivity than the hot water-insoluble residue, indicating that the compounds synthesized from acetate had been rapidly incorporated into macromolecular constituents. Both the hot water extract and an acid hydrolysate of the insoluble material were subjected to two-dimensional chromatography and radioautography. Virtually all radioactivity in the extract was present as aspartic acid. The hydrolysate contained radioactive aspartic acid, leucine, and proline. These results indicate that acetate is incorporated into specific amino acids which rapidly find their way into the protein fraction of the cells.

We have also investigated quantitatively the ability of sodium thioglycollate and sodium sulfide to furnish the sulfur required by S. bovis for growth. When the organism is grown in either the arginine or the ammonium medium, 2 to 5 mg. of sodium thioglycollate per 6 ml. culture supports maximum growth; only 0.1 to 1 mg. of $Na_2S \cdot 9H_2O$ are required

for best growth. Excessive amounts (3 to 5 mg.) of $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ retard growth, but no inhibition was observed with levels of sodium thioglycollate up to 5 mg. per 6 ml.

PLANS FOR FUTURE

(a) To confirm and extend the results obtained with radioactive acetate, (b) to investigate the biosynthesis of sulfur-containing amino acids in S. bovis by use of S^{35} labeled precursors and by isotopic competition, (c) to re-investigate the incorporation of C^{14}O_2 into amino acids to furnish a comparison of the roles acetate and CO_2 in amino acid biosynthesis.

CURRENT REPORTS AND PUBLICATIONS

J. M. Prescott, W. T. Williams, and Rae S. Ragland, "Influence of Nitrogen Source on Growth of Streptococcus bovis", Proc. Soc. Exp. Biol. Med. 102, 490 (1959).

THE POSSIBLE RELATIONSHIP OF STREPTOLYSIN "O" AND OTHER STREPTOCOCCAL ANTIGENS TO THE PATHOGENESIS OF RHEUMATIC FEVER.

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ASSISTED BY T. P. Auerbach and S. Keatinge

TASK NO. NR 134-295

CONTRACT Nonr 266 (32)

OBJECTIVES

(a) To purify streptolysin "O", and to study its effect on experimental animals; to determine whether the lesions produced may reveal an analogy to those in human rheumatic fever, and whether they may account for some of the lesions found in experimental animals by repeated streptococcal infections, (b) to isolate, characterize, and determine the biological activities of several apparently new streptococcal antigens found to be produced "in vivo" in humans during infections. To determine whether any of these may play a role in the pathogenesis of rheumatic fever.

ABSTRACT

Much of the effort during the past report period has been devoted to continuing purification of group A and group C electrophoretic and chromatographic fractions. Thus far, a total of 11 components have been well separated from the others, and have been shown to be immunologically distinct from each other. As previously reported, 4 of these have been identified as DPNase, DNase B, proteinase precursor, and streptolysin.

A fifth has now been identified as having the immunological specificity of the cell wall C carbohydrate. This fraction, present in a limited number of electrophoretic drip points, was not adsorbed on the CaPO_4 at low salt concentrations, and passed through the column completely. This fraction was active to lower dilutions immunologically, is precipitable with ammonium sulfate, is non-dialyzable and absorbs ultra violet light at 280 and 260 mμ. It has been found that this essentially single immunological entity shows the reaction of "identity" with a C carbohydrate preparation prepared by hot formamide extraction of the C203S cells. These findings indicate that the C carbohydrate is present in the culture supernates as a complex with a protein. Such observations are of much interest in view of the findings by Schwab, Cromartie, et al. that peculiar recurrent nodular skin lesions were produced by injection of an extract of sonic vibrated group A streptococcal cells. These investigators have found that the active principle which produces this lesion is apparently a rather large molecular species consisting essentially of a complex of the C carbohydrate and a protein. Because of this, several rabbits have been injected intradermally with the unadsorbed fraction mentioned above; the results thus far have been equivocal. Samples of the same fractions have been sent to Dr. Schwab to determine whether these materials injected into his strain of rabbit may not produce the nodular lesions he has described.

In addition, a fair amount of study has been applied to the separation of two components which are sensitive to antigen excess abolition of the precipitin bands. It has proven difficult to separate these, although

many preparations have been obtained which contain both of them and little else. Occasional fractions have been prepared in small amounts in which they are separated to a large extent from each other. Interest in these fractions is great, inasmuch as samples of highly purified scarlet fever toxin obtained from Dr. Soru in Rumania, and Dr. Hansen in Goteborg, Sweden, both showed reactions of "identity" with one or both of these "antigen excess" fractions. A sample of several fractions extremely rich in these components have been sent to Dr. Aaron Stock who has expressed willingness to assay them in rabbits for scarlet fever toxin potency.

Previous observations with continuous flow electrophoresis had indicated that the C203S strain of group A streptococcus secreted 12-13 extracellular antigens in vivo, detectable with human gamma globulin. As a result of subsequent chromatographic purifications, it became clear that appreciably more than this number were involved. Because of this, during the last 6 months, intense efforts made it possible to overcome certain technical difficulties and utilize immuno-electrophoretic methods in the study of these systems. These observations have revealed that the extracellular armamentarium of the streptococcus in vivo is even more complex than had been previously believed. The immuno-electrophoretic analyses now reveal that the number of antigenic secretory products which are detectable with human antibodies total at least 20 and possibly more. It can be seen from these data that the difficulties inherent in the separation of these components in such a complex system, therefore, has developed into a most imposing problem. It is felt that with adequate amounts of crude starting material, using the present technics, these separations can eventually be made, in order that the mechanisms of the pathogenesis of streptococcal disease be quite clearly evaluated.

Preliminary observations have been begun on the mechanism of death in rabbits from purified streptolysin "O". Early electrocardiographic studies indicate that death occurs as a result of acute cardiac electrical arrest. With moderately large doses, this may occur within several seconds after the injection has begun intravenously.

Four manuscripts are in preparation dealing with the further results obtained with purification of the streptococcal antigens.

Arrangements have been made with the Armed Forces Biological Warfare group at Fort Detrick to grow 500 liters of the C203S strain of group A streptococcus, using growth conditions we have found satisfactory for the secretion of these toxins. It is absolutely essential in work of this sort to deal with large quantities since proteins in dilute solution tend to deteriorate rather rapidly. It is hoped that with this large amount of group A culture, we shall obtain enough of some of the other highly purified fractions so that biological studies can be begun with them.

PLANS FOR FUTURE

Intensive efforts will be made to clarify the mechanism of cardiac toxicity of streptolysin "O" in living rabbits, using highly purified preparations. Electrocardiographic, blood pressure, and electro-encephalographic observations will be made. Control studies will be carried out with a number of the non-streptolysin extra-cellular streptococcal components. Further controls will be carried out with the highly purified

materials in which the streptolysin will be inactivated by various rather specific means. If adequate amounts of material are still available, histopathological correlative studies will be begun.

Experiments will be continued to separate and purify the extra-cellular streptococcal antigens detected with human antibodies. It is hoped that each will eventually be separated from the others, so that a complete knowledge may be had of the extra-cellular armamentarium of the streptococcus in vivo.

Immunoelectrophoretic analysis will continue so that the identity of the various arcs of precipitate can be established with the various well separated streptococcal components. This can serve as a standard reference system in such analyses.

THE EFFECT OF RAPID HEAT TREATMENT ON THE ANTIGENIC STRUCTURE
OF SALMONELLA TYPHIMURIUM

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ASSISTED BY E. Ward

TASK NO. NR 103-302

CONTRACT Nonr-1812(00)

OBJECTIVES

(a) An evaluation of the effect of rapid heat treatment as compared to that caused by formalin on the antigenic structure of the adenovirus Type I.

ABSTRACT

The investigation has been carried out essentially as outlined in the progress report for July 1959 with increased emphasis on the development of new techniques for the isolation, propagation and measurement of adenovirus Type I. An evaluation of new methods for testing for and measuring the immunological antigenicity of active adenovirus has been included along with our original study of the immunological antigenicity of rapid heat inactivated adenovirus.

The hemagglutination test of Friedman was evaluated for the purpose of supplementing or replacing in part the serum neutralization and complement fixation tests for measuring adenovirus induced immune responses. The difficulty in preparing the formalin fixed sensitized sheep red blood cells which consistently agglutinated spontaneously negated the practicability of applying this testing procedure in our investigation.

Prior to this report all of our vaccinations of active or rapid heat inactivated adenovirus had been carried out in rabbits. Since the original antibody titers by serum neutralization testing (SN) were rather low, a screening vaccination experiment in chickens and guinea pigs as well as rabbits was instituted. The choice of animal (chicken, rabbit or guinea pig) apparently makes little difference since at least one representative of each group inoculated with either active or rapid heat inactivated virus yielded a maximum SN titer of 1×10^5 /ml. Titers for all groups ranged from 1×10^2 /ml. to no response.

A preliminary investigation of poliomyelitis antibody production in mice by induced ascites was conducted since this method indicated promise in evaluating both active and inactivated adenovirus as an immunizing agent. Poliovirus was used since it produces cytopathic effect (CPE) rapidly in HeLa tissue culture. The strains of virus representing the three serological types were established attenuated ones and did not elicit antibody production in either the ascites fluids or the sera of vaccinated mice treated with "Freund's adjuvant" by SN tests using HeLa tissue culture. There is some question as to the ability of these attenuated strains to elicit an immune response in mice when introduced

parenterally.

Different tissue culture systems and techniques have been studied for the purpose of developing methods for increasing the speed of achieving end points in adenovirus titrations. Various established and primary tissue cultures have therefore been screened for the production of plaques and or CPE. While we have thus far been unsuccessful in producing plaques with adenovirus Type I in cultures of chick embryo, mouse embryo and guinea pig kidney, some definite effect has been observed in HeLa monolayers. The production of plaques as such has not as yet been accomplished. Plaque production is presently being investigated in cultures of chick kidney, guinea pig spleen, rabbit kidney and Earl's L cells as well as primary human fetal kidney.

We have been successful in cultivating adenovirus Type I in stationary tube and bottle cultures of established guinea pig spleen. Pools of this virus type have been prepared by cultivation in both HeLa and guinea pig spleen. Both pools were titrated before and after tissue-lay-down in tube cultures of both tissues. The results of these studies have led to the following conclusions: (1) the propagation of adenovirus in HeLa tissue culture results in a more sensitive titration by CPE in guinea pig spleen. It also brings about a much more rapid end point of virus CPE titer than by titration of HeLa propagated virus in HeLa tissue culture. (2) Inoculating adenovirus before tissue lay-down results in a more rapid development of CPE and a higher CPE₅₀ titer. (3) The propagation of adenovirus in guinea pig spleen results in development of a more rapid end point in HeLa tissue culture titrations. (4) A final conclusion is that the CPE produced by adenovirus Type I in guinea pig spleen is more pronounced and easily detected than in HeLa tissue culture.

PLANS FOR FUTURE

The immunological screening test will be expanded to include other animals not as yet tested and a repeat of the prior test with increased numbers for achieving a more consistent result will be carried out. The mouse induced ascites technique will be re-evaluated by testing known immunizing viruses as well as adenovirus. Plaque production and CPE studies will be continued with additional tissue types and the HeLa tissue effect produced by adenovirus will be further studied using acid, standard and alkaline agar overlays to select for a possible enhanced tissue effect.

CURRENT REPORTS AND PUBLICATIONS

(a) The CPE of Tissue Cultures Inoculated With Virus Before and After Tissue Lay-Down. I. Adenovirus. (To be published and offered for presentation at the 1960 SAB Convention in Philadelphia).

(b) The Immunological Antigenicity of Rapid Heat Inactivated Adenovirus. (In preparation for publication).

(c) The Propagation and Titration of Adenovirus Type I in an Established Guinea Pig Spleen Tissue Culture Cell Line (In preparation for publication).

SALT WATER FUNGI

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ASSISTED BY C. Edith Marks, Joe Levi and Steve Zellner

TASK NO. NR 103-305

CONTRACT Nonr-1811(00)

OBJECTIVES

(a) To study cellulolytic activity of the marine Ascomycetes and Deuteromycetes and associated degradation of lignocellulose tissue, (b) to examine interrelationships between the physiology of these organisms and the nutritional constituents of wood immersed in sea water, (c) to analyze the physiological determinants of their reproductive processes, (d) to investigate the ecology and general population dynamics of this fungal group as well as of other sea water types, including yeasts, present in marine sediments.

ABSTRACT

Ecological studies have shown significant differences in the dominant marine mycota in northern and southern environments, especially among the Deuteromycetes, including such widespread genera as Piricauda, Humicola, and representatives of the Helicosporae. Various genera and species, especially Antennospora quadricornuta, extremely prevalent in warmer oceanic areas, have not been found in such colder marine environments as Kodiak, Alaska, and Halifax, Nova Scotia.

An evaluation of growth and cellulolytic activity in over 30 helicosporous isolates from numerous separate marine localities shows considerable physiological dissimilarity within the group, indicating that there is a larger number of species of marine helicoid fungi than has been recognized heretofore.

Comparative tests of growth and cellulolytic activity of selected deuteromycetous species on balsa wood in yeast extract broth, prepared both in distilled water and in sea water, indicate wide salinity tolerances in this marine occurring group. This characteristic differs from that noted in the marine ascomycetous species tested which exhibit a definite requirement for sea water, and do poorly on media prepared in distilled water. Cellulolytic activity of filtrates of deuteromycete cultures from distilled water and sea water media were nearly identical. However, the glucose content of the reducing sugar fraction from individual species, and that from the activity of cultures grown on distilled water and sea water media, varied considerably. The significance of these variabilities, as well as of the cellulases, as indicating adjustment of fungi to the marine environment is being studied.

Selected species of marine Ascomycetes and Deuteromycetes, grown on Manila twine in culture vessels containing 0.1% yeast extract and sea water, have exhibited extensive cellulolytic activity accompanied by significant loss in the tensile strength of the fiber. Sterilization of the twine at 121 C. for 20 min., alone and in the yeast extract sea water medium, had no demonstrable affect on the physical properties of the Manila. The strength of the twine following fungal attack, designated

"residual tensile strength", varied considerably with infestation by different fungi. Tests of uni-clonal infested twine showed readings of from 74 to 183 lbs. at the breaking point compared with the 260 lbs. tensile strength of the check uninoculated twines. Considerable differences in residual tensile strength were observed between the 5 coils of the 50 ft. set used for each test, with maximal degradation, and often fungal reproduction, occurring in the surface, or partly exposed, upper one or two coils. The average ratio of the latter, compared with the completely submerged twine, fell between 1:1 and 1:2. Variability in fungal attack among coils in the same test flask was indicated by the range from maximal to minimal strength readings up to, or occasionally exceeding, 150 lbs.

Measurements of cellulolytic activity (Nelson-Somogyi method), using Walseth cellulose and CMC 50 T as cellulosic substrates for enzymatic activity, of the fungal filtrates have indicated: (a) In general, larger amounts of reducing sugars (RS) were produced by the deuteromycetous species than by the ascomycetous isolates. Isolates of the former group produced from 0.11-3.0 mg.RS/ml. on Walseth cellulose while species of Ascomycetes showed an RS range of 0.5-1.83 mg./ml. (b) The percent of glucose in the RS fraction from Walseth cellulose varied considerably, from 10 to 39% for the Ascomycetes and 17 to 100% for the Deuteromycetes examined. (c) Many of the cell free filtrates exhibited excellent RS production on CMC 50 T over a wide pH range, from 5.4 to 7.0. While a direct comparison of fungal enzymatic activity with residual tensile strength is not possible here, further studies in this direction are in progress. Nevertheless, positive evidence is presented demonstrating the ability of these lignicolous marine fungi to produce extensive degradation of cordage, thus indicating ability to attack a native lignocellulose complex.

PLANS FOR FUTURE

Work in progress or contemplated for the near future includes (a) further chromatographic analyses of the soluble extractives from selected woods, as related to fungal growth and reproduction, and evaluation of the effects of salt water immersion on woods under aseptic conditions, (b) continued studies of cellulolytic activity including enzymatic and anatomical investigations of the degradation and loss of strength of lignocellulose substrates including Manila, (c) nutritional and physiological tests involving, studies of minimal requirements, relationship of reproduction to cellulase production, and response to salinity and temperature, (d) studies of marine yeasts and pelagic fungi, with emphasis on the physiological characteristics of selected representatives of this mycota.

CURRENT REPORTS AND PUBLICATIONS

(a) S. P. Meyers and E. S. Reynolds (1959), "Growth and cellulolytic activity of lignicolous Deuteromycetes from marine localities." *Canad. J. Microbiol.* 5: 493-503.

(b) S. P. Meyers and E. S. Reynolds (1959), "Effects of wood and wood products on perithecial development by lignicolous marine Ascomycetes." *Mycologia* 51: 138-145.

(c) S. P. Meyers and E. S. Reynolds (1959), "Cellulolytic activity in lignicolous marine Ascomycetes." *Bull. Mar. Sci. Gulf and Caribbean* 9: 441-455.

(d) S. P. Meyers and E. S. Reynolds , "Occurrence of lignicolous fungi in northern Atlantic and Pacific marine localities."

FUNCTION AND METHOD OF DNA LEAKAGE BY MICROBIAL CELLS

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ASSISTED BY W. Guschlbauer and M. Durkin

TASK NO. NR 103-307

CONTRACT Nonr-1582(02)

OBJECTIVES

(a) To determine the mechanism by which highly polymerized DNA gains entrance but into the environment from viable bacterial cells. (b) To determine the types of enzymes that are present in slime cultures which act on highly polymerized DNA. (c) To determine whether human tissue carcinoma DNA and DNases reacts similarly to the microbial DNA and DNases.

ABSTRACT

Extractions performed on the cell free culture medium and the cells of Pseudomonas aeruginosa yielded the presence of two different DNases. The extracellular DNase has a pH optimum of 5 and apparently no requirement for divalent ions. However the enzyme was stimulated in activity by the presence of the combined presence of magnesium or calcium ion and citrate ion. The intracellular enzyme of Pseudomonas aeruginosa is similar to the extracellular enzyme in every respect except its pH optimum. The pH optimum was found to be at 6 rather than 5. The possibility still exists that the enzymes extracted may have also contained as coprecipitated impurities another type of DNase with activity in the alkaline pH. However no method of extraction was successful in detecting these enzymes in any quantity.

Extractions on a Brodir's adenocarcinoma for DNA and DNases yielded interesting data. No highly polymerized DNA could be found in the tissue. However, enzyme extraction yielded an active preparation which contained possibly two different DNases. One DNase had a pH optimum at 6 with no divalent ion requirement and the other DNase, in much higher concentration, was active at pH 7-7.5 and had a requirement for magnesium ion.

Other carcinoma extractions did not yield highly polymerized DNA but only the depolymerized moieties.

PLANS FOR FUTURE

(a) To determine the type of enzymes that affect the DNA of Pseudomonas P-16, another slimer, and the effect of the extracted DNA's on the DNases of other origins (b) To determine the relative abundance of the DNases in other neoplastic tissue and determine whether these are reciprocity of activity or highly polymerized DNA from slime bacteria.

CURRENT REPORTS AND PUBLICATIONS

- (a) DNases of Pseudomonas aeruginosa and adenocarcinoma tissue.
M. S. Thesis, W. Guschlbauer. 1959
- (b) Deoxyribonucleases in an adenocarcinoma. In press
- (c) Deoxyribonucleases from Pseudomonas aeruginosa. J. Bact.
In press

THE ROLE OF PUTRESCINE IN MICROBIAL METABOLISM

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ASSISTED BY Matthew R. Purvis

TASK NO. NR 103-317

CONTRACT Nonr-595(06)

OBJECTIVES

To determine the essential function of putrescine and related growth factors in the metabolism of Hemophilus parainfluenzae.

ABSTRACT

Previous reports on this project have established the association of putrescine and other naturally occurring diamines and polyamines with bacterial nucleic acids. Spermine-nucleic acid complexes are quite resistant to enzymatic hydrolysis by nucleases and experimental work to determine the effect of polyamines on nucleic acid turnover in growing and resting bacteria has been undertaken. The nucleic acid which is synthesized by bacteria during incubation in complete media containing chloramphenicol has been reported to be unstable, presumably because of rapid enzymatic hydrolysis. We have studied nucleic acid biosynthesis and turnover in chloramphenicol inhibited systems in the presence of spermine. More nucleic acid accumulates in cells incubated in the presence of spermine as compared with controls incubated without spermine. The effect is reproducible and in the order of a 25% stimulation of nucleic acid synthesis. When the cells which have carried out nucleic acid synthesis in the presence of chloramphenicol are washed free of the antibiotic and resuspended in growth media, an initial degradation of the cellular nucleic acid occurs followed by a recovery of the nucleic acid with continued incubation. When these experiments were carried out in media containing spermine, the initial loss of nucleic acid did not occur. We believe that these observations might prove useful in future investigations aimed at the characterization of unstable nucleic acids.

PLANS FOR FUTURE

We will continue the experiments on the effect of spermine and precursor amines such as spermidine, putrescine, and 1,3-propanediamine on nucleic acid metabolism. The two principle approaches to the problem will be: (a) the molecular form of amines in microorganisms and (b) the role of amine-nucleic acid complexes in cellular physiology.

CURRENT REPORTS AND PUBLICATIONS

(a) E. J. Herbst and B. P. Doctor, (1959), "Inhibition of Ribonucleic Acid Degradation in Bacteria by Spermine." J. Biol. Chem., 234, 1497-1500.

(b) D. L. Keister and E. J. Herbst, (1959), "The Role of Spermine in Stabilizing Bacterial Ribonucleic Acid." In preparation for J. Biol. Chem.

FACTORS AFFECTING THE PERMANENT EFFICIENCY OF
THE ANTIBODY-PRODUCING MECHANISM

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TASK NO. NR 130-323

CONTRACT Nonr-233 (40)

OBJECTIVES

(a)To ascertain the validity of the claim that the antibody-producing mechanism is suppressed in a highly specific manner by the introduction of antigen into the body of the fetal or newborn animal, (b)To determine conditions favorable for immunological suppression, (c)To determine whether such suppression occurs when the antigen enters the fetal body by way of the placenta or other fetal-maternal exchange organs.

ABSTRACT

Earlier work demonstrated that heterologous serum proteins pass from the blood of the pregnant rabbit to that of the fetus. Both human and bovine gamma globulins and albumins were used. These proteins were found in various fetal fluids as well as in the blood serum, and the concentrations were definitely higher in the fetal blood during the last few days of gestation than in earlier stages.

The question was, might such transferred proteins act as effective antigen in the establishment of immunological inhibition? Pregnant rabbits at 20-26 days of gestation were given various doses of antigen which was shown to pass to the young. When challenged for inhibition at the age of 7 months, these animals showed no evidence of inhibition. Similarly, no inhibition was obtained in the young of females injected on the 28th day of gestation.

The above animals were challenged for their capacity to produce specific antibody at the age of 7 months. Since we know that inhibition wears off in many animals, the experiment was repeated but animals were challenged at 4 weeks and at seven weeks after birth. Immunological inhibition was now clearly evident as shown by the longer persistence of antigen in experimentals as compared with controls, and the actual absence of humoral antibody in experimentals. Both the precipitation and tanned red cell tests were used.

In similar experiments with chickens it was noted that animals inhibited against bovine serum albumin also showed a moderate degree of inhibition for human gamma globulin. This raised the question, do antigenic determinants for antibody production play an equivalent role in immunological inhibition or do they display different behavior? In other words, if the antigenic determinants play an equivalent role in both antibody production and inhibition, then the degree of cross re-

action should be equal to the degree of cross inhibition. In order to make such a comparison normal rabbits were injected with bovine gamma globulin and the anti-BGG was shown to react with BSA and human gamma globulin in the order of 3-6% of the homologous reaction. However, neonatal rabbits injected with BGG showed a 70-80% reduction in antibody reactive with BSA and HGG.

Chickens showed a quite different situation. Newly hatched chicks injected with BGG showed no inhibition for BSA or HGG when challenged at maturity, although they were clearly inhibited for the homologous antigen. It is, therefore, evident that antigenic determinants are quantitatively unequal in their effects upon inhibition and antibody production.

PLANS FOR FUTURE

Current work is concerned with the inhibiting effects of complex, natural antigens as distinct from the simple proteins used hitherto. Vaccines prepared from whole trypanosomes are being used to ascertain whether a state of immunological inhibition can be established with respect to these protozoa. We have found that injection of trypanosomes into 10-day old rats results in an immune state. Now rats of younger age are being tested. The normal rat quite invariably becomes immune to infection with the trypanosomes; the latter seem to have little ill effect upon the host. If the animals can be made immunologically tolerant (suppressed), it may be possible to render the trypanosomes pathogenic.

Work is also under way on the resistance of mice to various tumors. This problem also involves the supposed antigenic individuality of tumors as distinct from normal tissues. Preliminary results have shown that it is possible to distinguish the Ehrlich ascites tumor from the normal tissue of the C3H mouse.

CURRENT REPORTS AND PUBLICATIONS

- Timourian, H. and Schechtman, A.M. 1960 The specificity of immunological depression. Federation Proceedings, 1960. Submitted abstract. Oral report will be presented at Chicago meetings of the Federation.
- Levi, E., Schechtman, A.M., Sherins, R. and Tobias, S. 1959 Tumor specificity and immunological depression. Nature, 1959. This has been published but no reprints have as yet arrived.
- Hirat, A. and Schechtman, A.M. 1960 Studies on immunological suppression in chickens. J. Immunol. Ms. submitted.

THE ROLE OF CONCURRENT OR SYNERGISTIC EFFECT BETWEEN VIRUS
AND BACTERIA IN ENTERIC INFECTIONS

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ASSISTED BY Mrs. Adamadia D. Davis

TASK NO. NR 103-325

CONTRACT N7onr-33006

OBJECTIVES

To isolate and propagate filterable agents involved in enteric infections.

ABSTRACT

Eight additional cytopathic bovine virus isolates have been recovered in bovine kidney cell cultures. Three of these were recovered from feces of cows and calves in herds suffering losses from white muscle disease (myopathy) among the young calves, two from spleen and hemorrhagic adrenals of very young calves suffering peracute death and two from the respiratory tracts of calves with respiratory disease. One of the virus isolates recovered from the respiratory tract has been identified as infectious bovine rhinotracheitis (IBR) virus.

In cell culture neutralization tests, bovine enterovirus (BEV) isolates were inhibited by IBR rabbit immune serums but IBR virus was not inhibited by BEV rabbit immune serums. This "one way" relationship between BEV and IBR viruses was more pronounced in the serum dilution-constant virus system.

BEV isolates caused fetal deaths when inoculated into pregnant guinea pigs during the second half of gestation. The dead offspring showed marked acute serous hepatitis and also some cardiac damage.

The duration of BEV shedding in feces subsequent to infection, and specific neutralizing antibody levels before, during, and after virus shedding have been studied in cattle on infected premises. BEV is present in the contents of all segments of the large intestines and also in rumen content of bovine virus shedders.

PLANS FOR FUTURE

1. Study of the origin of the BEV in the gastrointestinal tract of cattle during the shedding period.
2. Further study of disease-producing capacities of BEV isolates in laboratory animals.

CURRENT REPORTS AND PUBLICATIONS

(a) T. Moll and A. D. Davis (1959), "The serological relationships between bovine enteroviruses (BEV) and infectious bovine rhinotracheitis (IBR) virus." Submitted to Jour. Vet. Res. for publication.

STUDIES ON THE FRACTIONATION OF PNEUMOCOCCAL TRANSFORMING FACTORS

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ASSISTED BY Leatrice Lowi

TASK NO. NR NR 103-327

CONTRACT Nonr-266(40)

OBJECTIVES

To investigate the fractionation of pneumococcal transforming deoxyribonucleic acid (DNA) by ion exchange chromatography.

ABSTRACT

The fractionation of pneumococcal DNA by chromatography on the substituted-cellulose anion-exchanger, ECTEOLA, has been continued. The difference in elution profiles between the DNA from the donor strain and the DNA from the recipient strain, has been confirmed. New strains of pneumococci have been prepared from the recipient strain in which the markers have been introduced singly, and in known sequence. DNA preparations have been obtained from these strains, and their chromatographic profiles are being determined. It is thus hoped that the profile may be correlated with genetic constitution.

The effect of various polyamines, e. g., putrescine, piperidine, di-aminopropane, cadaverine, spermine, and spermidine, on the transforming reaction was studied. Low concentrations of the polyamines protected transforming DNA, in solution in distilled water, against inactivation, but did not have any effect on transforming DNA in solution in 0.85% saline.

As part of a continuing search for new markers, it has been found possible to transform to amphomycin resistance. This marker does not appear to be linked to any of the other markers under investigation.

PLANS FOR FUTURE

To continue and expand the studies along the following lines:
a) investigation of the relationship between the chromatographic profiles and the hereditary characteristics of the newly-isolated strains from which the DNA was obtained; b) studies of the inhibitory activity of fractions of DNA prepared from the receptor strain.

CURRENT REPORTS AND PUBLICATIONS

(a) S. M. Beiser, H. B. Pahl, H. S. Rosenkranz, and A. Bendich (1959), "Studies on the fractionation of transforming deoxyribonucleic acid of pneumococcus. I. Recovery and dose-response relationships." *Biochem. Biophys. Acta*, 34, 497-502

(b) S. A. Ellison and S. M. Beiser (1960), "The effect of ultraviolet irradiation on pneumococcus transforming factor." *Rad. Res.*, In press.

THE CHEMICAL NATURE OF VIRUS PROTEIN

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and Technology

ASSISTED BY Richard P. Oates

TASK NO. NR 103-349

CONTRACT Nonr-1869(00)

OBJECTIVES

(a) To confirm the amino acid composition of T2 phage protein, and (b) to determine the degree to which labeled aspartic and glutamic acids are incorporated into T2 protein from the growth medium.

ABSTRACT

The newer system for column fractionation as stated in the last report, required several months for standardization procedures. Several virus hydrolyzates have been separated on the IR-120 column, and resolution has been good. Results obtained on hydrolyzates of T2 phage tend to confirm the qualitative amino acid composition reported by Fraser (J. Biol. Chem., 227, 711, 1957). Quantitative results are not quite the same as those reported by Fraser, but these data have not been substantially confirmed.

PLANS FOR FUTURE

(a) To substitute a battery of known amino acids for the casamino acids in Fraser and Jerrell's medium, and (b) to measure the incorporation of C-14 labeled aspartic and glutamic acids into T2 protein.

A STUDY OF FACTORS INFLUENCING VIRAL PARASITISM

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ASSISTED BY Joan Thompson

TASK NO. NR 130-352

CONTRACT Nonr-401(23)

OBJECTIVES

(a) To evaluate the relationship between age of host and ability of virus to persist in recoverable amounts. (b) To analyze the substances in colostrum that adjust the host-virus relationship in favor of the host, and (c) to define nutritional factors which permit the host to control viral parasitism.

ABSTRACT

Hog cholera virus, partially modified by 16 passages in rabbits, was inoculated into disease-free pigs. Pigs over 3 months of age became immune and grew in a normal way. In certain pigs under 3 months of age virus persisted in recoverable amounts and caused stunting.

Iron deficiency anemia is common among young mammals, especially swine. Our initial studies, therefore, were directed toward the effect of iron on the host-virus relationship which permitted virus to persist and cause stunting in the younger pigs. It was found that pigs made deficient in iron were stunted when given modified hog cholera virus, whereas, those with normal iron stores continued to grow and became immune. It appeared that iron deficiency was a factor in the control of viral parasitism by pigs.

Studies of cytochrome C seemed indicated for further clarification of this failure of iron deficient pigs to control viral parasitism and pigs made deficient in iron showed a lower level of cytochrome C in their livers whether 6 weeks or 12 weeks of age than littermates given iron. The amount of cytochrome C at 12 weeks of age, however, was 65-70% greater than at 6 weeks of age when compared within respective treatment groups. This finding lends support to the concept that iron deficiency creates disturbances in tissue metabolism and has an effect on viral parasitism.

PLANS FOR FUTURE

Studies on cytochrome C will be continued. Skin grafts as a measure of immunological tolerance will be substituted for viruses in some studies.

FACTS INFLUENCING TISSUE-VIRUS INTERACTIONS, WITH
SPECIAL EMPHASIS ON IN VIVO TISSUE CULTURE

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TASK NO. NR 103-371

CONTRACT Nonr-840 (07)

OBJECTIVES

(a) To develop improved techniques for the establishment of in vivo tissue and organ cultures, (b) to extend the study of virus infections in tissues from susceptible animals transplanted to resistant animals, (c) to study the dynamics of virus multiplication in such systems and to evaluate the factors determining the different responses of the transplant to the virus.

ABSTRACT

Investigations with influenza virus infection in transplanted chick embryo tissues in the brains of rats and newly hatched chicks revealed additional information. Rat brains bearing chick embryo transplants inoculated with PR-8 influenza virus with an infectivity titer of 10^{-8} , yielded a level of 3.2 logs of virus per 0.1 ml of a 20% suspension. It is interesting that the histologic appearance of the infected transplants in the rat brain was about the same as that of uninfected controls. There was no obvious damage caused by the virus. The same virus, when injected intracerebrally into newly hatched chicks, gave the following results: Chick brains containing no transplant showed virus levels of 1 or less logs following intracerebral inoculation of undiluted virus into the brain. Chick brains containing an embryonic transplant yielded 3.5 logs of virus following such inoculation. It was also noted that a lymphocytic response previously mentioned in connection with transplantation to the brain of chick tissue elements was frequently elicited by the inoculation of influenza virus (virus grown in chick embryos).

The transplantation of minced chick tissue into rat brain was associated with survival or growth of normal chick embryonic elements, predominantly cartilage, skin, and mesenchyme, though other structures were also evident. These observations were made in animals conditioned by means of Cortisone and x-ray. When such minces were first exposed for a few minutes to Rous Sarcoma virus and then implanted, the results consisted of extensive developments of Rous Sarcoma tumor in the rat brain. Thus, the

heterologous transplant permits the growth of a malignant lesion in a normally resistant animal species. During this work it came to our attention that in their report "The Transplantation of Tissue Brain Between Zoological Classes" (Can. Res. 13, 64 (1953), Allbrink and Greene demonstrated sarcomatous changes in chick tissue bathed in Rous Sarcoma virus prior to transplantation to mouse brain. These authors used non-conditioned animals. In contrast, our results indicated that conditioning of animals was required, only occasional tumors arising in non-conditioned animals. Embryonic implants which did not form Rous Sarcoma showed slightly more degeneration and necrosis and slightly more host response in the non-conditioned than in the conditioned rats. Sarcomatous growth was also obtained in chick brains receiving homologous embryonic transplants pre-treated with Rous Sarcoma virus. There was considerably more necrosis of these implants (especially the sarcoma) than in the rat brains. More recently, the exposure of chick fibroblasts (obtained by trypsinization of the embryo or from primary tissue cultures) to Rous Sarcoma virus, produced abundant tumors in rat brains. This technique is associated with a lower animal mortality, and should furnish a better basis for quantitative studies.

In connection with in vitro tissue culture work, several lines of cells were established. A rabbit liver culture and three calf-kidney lines showed the characteristics of tissue culture transformation with respect to morphology and to susceptibility to polio and Cocksackie viruses. By contrast, cultures of calf trachea, lung and bronchus have become established without apparent alteration, either morphologically or with respect to susceptibility to polio virus. When tested by our rat brain transplantation techniques, the "transformed" cultures (rabbit liver and calf kidney) produced tumors, whereas the seemingly unaltered cells gave rise to small nests of normal-appearing cells.

PLANS FOR FUTURE

The plans are essentially as expressed by the objectives above. More emphasis will be given to the problem of re-differentiation and organization in the transplant.

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THE EFFECT OF MULTIPLE ANTIGEN STIMULATION UPON ANTIBODY
RESPONSE OF ANIMALS--A QUANTITATIVE STUDY

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TASK NO. NR 103-390

CONTRACT Nonr-1202(12)

OBJECTIVES

(a) To study the effect of simultaneously injecting 2 (or more) antigens of different molecular size on antibody formation, (b) to determine if a presensitization to one antigen will affect the response of a simultaneous injection of 2 or more antigens, (c) to correlate varying dosages of each antigen with the type of response elicited by simultaneous inoculations.

ABSTRACT

The simultaneous injection of 2 good antigens (bovine serum albumin and human gamma globulin) into chickens results in a lowered response to the antigen of lower molecular weight if the amount of the inoculation is less than optimal. On the other hand recent findings indicate that if an optimal dosage is used then there is no difference in the response elicited by either antigen. These seemingly contradictory results have necessitated additional experiments to verify these findings.

Chickens were given a primary simultaneous inoculation of the 2 antigens followed by the secondary injection of the same 2 antigens. The data seem quite definite that a secondary type of response resulted to the larger molecule (globulin) and a delayed response to the albumin. The peak titer appeared at 5 days for the globulin and at days 9 to 10 for the albumin. Furthermore the titers for the secondary response were much greater for the globulin. These results are not conclusive as the variation in antibody response is considerable. Additional animals are to be inoculated in order to be able to get sufficient data for statistical analyses.

PLANS FOR FUTURE

The results to date emphasize the need for use of a larger number of animals. This is necessary because of the great variation in response. The immediate plan is to attempt to repeat some of the experiments described in the 'abstract'. An experiment has already been started in which chickens have been given a simultaneous injection of an optimal dose of bovine albumin and human gamma globulin. The secondary injection will be similar. This will probably be followed by tertiary inoculation. A second experiment has also been started. Chickens have been sensitized with either bovine serum albumin or human gamma globulin. A period of several months has passed and these animals are to be injected with both antigens. The purpose of this is to add to the meager amount of data we have accumulated on this particular problem.

A STUDY OF THE EFFECT OF CORTISONE ON THE GROWTH OF VIRUSES
IN TISSUE CULTURE

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ASSISTED BY

TASK NO. NR 103-393

CONTRACT Nonr-668(13)

OBJECTIVES

To determine the effect of cortisone on the growth of Psittacosis virus (strain 6BC) in cultures of mouse fibroblast cells (strain L) with particular reference to the diverse effects produced by cortisone on the virus growth curve.

ABSTRACT

Studies to date on the effect of cortisone on the growth of Psittacosis virus in L cells have shown cortisone to produce two apparent effects. The first is an initial suppression of virus growth, followed later by an enhancement of virus growth. Recent studies have been concerned with an analysis of this phenomenon. The effect of cortisone in enhancing virus growth was investigated by comparing virus growth in cortisone-treated and control cultures in relation to the number of cells present in both systems at given time intervals. The result of this study indicated that cortisone protects the cells from virus cytopathic effects resulting in a significantly higher number of cells capable of continuing the synthesis of virus than are found in the control cultures at the same time period. It would thus appear that cortisone does not affect the cell in terms of the amount of virus that can be produced, but rather to protect the cell from some of the consequences of virus synthesis. A study of the initial effect of cortisone, that of apparently suppressing virus growth, was made by comparing virus titers in both cells and tissue culture fluids during this early time period. The results of this study showed that the titers of virus in the cells were the same in the cortisone-treated and control cultures, although the virus titers of the fluids of cortisone-treated cultures were markedly lower than those of the controls, indicating that cortisone has no effect on virus synthesis in this system but suppresses or delays virus release from the cells.

In association with this study, the virus assay method was examined with the intent of obtaining more reliable estimates of the LD₅₀. The virus assays have been carried out using the single dilution method of Golub (J. Immunol., 59, 71, 1948). This method of determining LD₅₀ end-points was found to be inadequate on occasion because of what appeared to be a variation in the susceptibility of the eggs. This variability has been found to occur most frequently during the summer months. During this time, a series of assays of a standard virus preparation were carried out and the results analysed by both probit and rankit methods. This study

showed that the embryonated hens' eggs differed significantly from week to week in terms of their over-all susceptibility to infection with Psittacosis virus, and that on occasion marked variation in susceptibility could occur within a single group of eggs. The use of a standard curve, prepared each week, and rankit analysis has made it possible to relate virus titers within an experiment when egg susceptibility is variable over the time period virus assays are being carried out.

PLANS FOR FUTURE

Studies of the virus growth curve following the initial suppression of virus release by cortisone will be carried out in an attempt to find an explanation for the parallel rates of virus growth in cortisone-treated and control cultures occurring for a 48-hour period.

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STUDIES ON THE UTILIZATION OF COMPOUNDS FOUND IN NATURE BY MARINE BACTERIA

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TASK NO. NR 103-398

CONTRACT Nonr-2337(00)

OBJECTIVES

(a) To isolate marine bacteria which can utilize uronic acids and alginic acid, (b) to determine the effects of the cations of sea salt on the metabolism of the bacteria, and (c) to study the intermediary metabolism of the acids by the marine isolates.

ABSTRACT

An obligately marine bacterium (M11) has been found to grow in a medium containing ammonium phosphate, sodium glucuronate, magnesium sulfate, and varying concentrations of potassium and sodium chlorides. Growth was observed to be evenly proportional to the concentration of NaCl over the range 0.06 to 0.25 M with KCl constant at 0.01 M. With NaCl constant at 0.25 M, growth was evenly proportional to the concentration of KCl over the range 0.0 to 0.002 M. Increasing the concentration of KCl to 0.01 M did not increase or decrease the growth response. Lithium, rubidium and cesium ions did not replace either sodium or potassium ions in the nutrition of M11.

When attempts were made to use sucrose as a possible metabolically inert osmotic replacement for sodium ions, it was found that M11 oxidized this substrate, consuming 4 μ moles of O_2 per μ moles of sucrose and 2 μ moles of O_2 per μ mole of each constituent hexose. An active sucrase was observed in broth grown cells.

Additional studies were carried out with alginolytic bacteria. Alginomonas alginica, which rapidly oxidized mannuronic acid, did not metabolize D-lyxose (the pentose which results from the decarboxylation on this uronic acid) or any other pentose. Fructuronic acid was not metabolized and appears not to be involved in the metabolism of mannuronic acid. An unidentified intermediate has been trapped with hydrazine from reaction mixtures of cell-free extracts and mannuronic acid.

An unidentified alginolytic bacterium which oxidizes mannuronic acid constitutively has been isolated from soil enriched with sodium alginate. The isolate grows well in sea water medium and is psychrophilic. No exocellular alginase could be demonstrated in supernates from cultures of this isolate. The bacterium, like A. alginica, does

not metabolize either galacturonic or glucuronic acid.

PLANS FOR FUTURE

(a) To study the effects of sodium and potassium ions on the induction of glucuronate "permease" and glucuronoxidase in M11 with the aid of C¹⁴-labeled glucuronate (b) To isolate and study the alginase of Alginomonas alginica and the unidentified soil isolate (c) To identify the metabolite of mannuronic acid dissimilation trapped by hydrazine.

CURRENT REPORTS AND PUBLICATIONS

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HOST-PARASITE INTERRELATIONSHIPS IN RICKETTSIAL INFECTIONS

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TASK NO. NR 103-399

CONTRACT Monr-538 (10)

OBJECTIVES

- (1) To identify the host-independent metabolic activities of Coxiella burnetii.
- (2) To study the contribution of rickettsial-infected cells to the physiology of C. burnetii.
- (3) To utilize biochemical information of C. burnetii to prepare a medium for propagation of the rickettsial agents.

ABSTRACT

Purified suspensions of C. burnetii are disrupted in a Nossal shaker. The cell-free material, designated as the "enzyme preparation", apparently catalyzes the reduction of TPN^+ in the presence of glucose-6-phosphate (G-6-P). Thus, 15 μ moles phosphate G-6-P, 10 μ moles MgCl_2 , 0.4 mg TPN^+ , in Tris buffer, pH 7.4, final volume, 1.45 ml, incubated with the enzymes at 35 C, produced a Δ O. D. at 340 m μ of 0.15 in 60 minutes. The production of 6-phosphogluconate was demonstrated chromatographically. It appears that the following reaction occurs:



Enzyme preparations from Phase 1 and Phase 2 show no significant variation in activity.

Cell-free preparations of C. burnetii also possess an active isocitric dehydrogenase, which at pH 7.4 produces a Δ O. D. 340 m μ of 1.0 in the presence of TPN^+ . C. burnetii uses carbamyl phosphate (CP). Employing CP^{32} , glucose, ADP and hexokinase, a Ba-soluble, EtOH insoluble, P^{32} containing fraction has been isolated which tentatively appears to be G-6-P.

PLANS FOR FUTURE

- (1) Study the synthesis of purines and pyrimidines by C. burnetii, employing radioisotope labeled precursors including CP, aspartate, 1-pyrophosphoryl-S phosphoribose, glycine.
- (2) Quantitatively examine the amino acid composition of C. burnetii.
- (3) Continue efforts to prepare an in vitro medium for the rickettsiae.

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TRANSFORMATION OF CAROTENOIDS BY MARINE BACTERIA

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TASK NO. NR 103-404

CONTRACT Nonr-2439(01)

OBJECTIVES

To determine the extent and nature of bacterial influences on carotenoids in the marine environment.

ABSTRACT

Investigations to determine the extent to which beta-carotene is attacked by marine bacteria under aerobic and anaerobic conditions have continued. Because of the high rate of autoxidation of carotenoids, and their apparent resistance to microbial degradation, it was not possible to detect any bacterial oxidation. Preliminary oxidation studies, using a Warburg respirometer, have revealed an inhibitory effect of beta-carotene on bacterial respiration. Inhibition of bacterial respiration by carotene is currently being studied to determine whether this inhibition involves specific enzymes, is caused by trace impurities in carotene preparations, or is a surface effect.

Some of the inconsistencies in results of experiments to determine the ability of marine bacteria to decompose carotenoids under anaerobic conditions have been explained. It has been found that carotene, which is dispersed in sea water with surface-active agents, is readily adsorbed on clay or silt particles (sediments) when these are used to inoculate carotene media. The adsorption may have two effects: (1) irreversible adsorption of some of the carotene, and (2) catalytic oxidation of carotene by traces of oxygen in the anaerobic systems. Both effects lower the concentration of carotene which can be recovered from the media. These effects of sediments are also obtained with autoclaved materials, and can not be the result of bacterial action. Suspensions of bacteria, when inoculated into carotene - sea water media, also adsorb some of the carotene, and probably are responsible for losses of carotene similar to those observed with autoclaved sediment samples.

When bacteria are supplied with another carbon source, and incubated with dispersed carotene, the carotene is generally protected from autoxidation by the metabolic activity of the bacteria. Hence, it appears that marine bacteria in sediments aid in protecting the deposited carotenoids, rather than decomposing them.

The adsorption of beta-carotene by marine sediment samples, and the possible protection of carotenoids by bacterial activity, prompted an investigation of the distribution of carotenoids in sediments. From the

limited number of core samples studied, a pattern of distribution of carotenoids seems to be emerging. The distribution varies with the type of sediment, and with the depth of the samples. Sandy sediments are lower in carotenoids than are clay and silt sediments. Carotenoid concentrations are generally highest near the surface, decrease rapidly with depth, reach a minimum level, and then rise again and remain fairly constant with depth. Rapid achievement of anaerobic conditions in the sediments seems to be essential for the preservation of the carotenoids. Bacteria in the upper six inches of sandy sediments probably contribute to the rapid establishment of anaerobic conditions. The increase in carotenoid concentrations with depth in the clay or silt sediments can be explained on the basis of the observed adsorption effects.

Studies on the proteolytic breakdown of the chromoproteins, phycoerythrin and two phycocyanins, of Porphyra leucosticta (Thuret) have shown that phycoerythrin is much more resistant than is phycocyanin to this type of attack. Crystalline trypsin digested the three chromoproteins in the same manner, and at rates similar to those which have been observed and reported earlier with whole bacteria, bacterial filtrates, and crude marine bacterial enzyme preparations.

PLANS FOR FUTURE

(a) Warburg studies to determine the nature of the inhibition of marine bacterial respiration by beta-carotene; (b) carotenoid decomposition studies will be expanded to include the possible decomposition by marine bacteria of hydrocarbons other than the carotenoids; (c) analyses of the carotenoid composition of additional sediment cores; (d) the difference in resistance of phycoerythrin and phycocyanin to proteolytic breakdown will be studied further.

RESEARCH ON THE LABORATORY DIAGNOSIS
OF VIRAL DISEASES

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TASK NO. NR 103-413

CONTRACT Nonr-2267(00)

OBJECTIVES

The development of reliable and specific reagents for the diagnosis of viral infections, particularly by the use of complement-fixation technique.

ABSTRACT

Work conducted with the aid of the equipment loaned under this contract continued to involve two groups of viruses.

1. Influenza. Past studies were concerned with the use of strain-specific (anti-V) guinea pig sera in the differentiation of influenza virus strains by complement fixation tests and with the rapid identification of new isolates. Strain-specific V antigens were employed for the serodiagnosis of influenza and for measuring antibody responses to vaccination with excellent results. An analysis of the breadth of anti-V responses has given the following results. Only in very young children is a monospecific response measurable; i.e., on presumably first contact with an influenza virus. Even under these conditions, if the disease is severe antibodies to V antigens of other strains may be formed. A correlation of results to the age of the donor frequently revealed antibodies to the dominant antigens of strains to which the individual could not have been exposed during his life span. A single severe infection or multiple past exposures broaden the anti-V responses, a fact which must be considered in the evaluation of the "original antigenic sin theory".

2. Poliomyelitis. Progress has been made in the separation of the type-specific heat-labile (N) and heat-stable (H) antigens on ion exchange columns. Clean-cut separations were obtained in some experiments, but the results have not been regularly reproducible. It seems that the naturally occurring H differs from the artificially produced H (56° for 30-60 minutes).

PLANS FOR FUTURE

The work will continue along the lines indicated. In the influenza studies efforts will be made to correlate heterologous anti-V responses to neutralizing antibodies.

GENETICS AND PHYSIOLOGY OF THE DIAUXIE PHENOMENON
AND ACTIVE TRANSPORT IN BACTERIA

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ASSISTED BY N. Killeen

TASK NO. NR 103-429

CONTRACT Nonr-624(07)

OBJECTIVES

(a) To determine by a study of diauxie resistant mutants the nature, genetic and biochemical, of the diauxie phenomenon, (b) to determine the characteristics of the "permease" involved in adaptation to citrate utilization, (c) to investigate the linkage relationships and the possible biochemical differences in the functioning of "non-identical alleles" involved in the synthesis of a single enzyme.

ABSTRACT

1. The correlation between decreased growth rate, increased acid phosphatase activity, and diauxie resistance and the deficiency of a carbohydrate non-utilizing mutant ($C^{+}dg^{S}$) of Salmonella typhimurium has suggested two hypotheses to explain the glucose diauxie phenomenon which we are testing. (1) The phosphorylated and non-phosphorylated intermediates in glucose metabolism, up to, but not including, Krebs cycle compounds and those produced from Krebs cycle compounds, are the repressors of induced enzyme formation. (2) Inorganic phosphate is limiting during glucose metabolism. The inorganic phosphate may be required for the synthesis of specific factors (e.g. ribonucleic acid) necessary for the synthesis of inducible enzymes. The liberation of inorganic phosphate by the phosphatase would then enable this synthesis to occur. In order to determine whether the increased acid phosphatase activity as measured with cell extracts is of significance intracellularly, we have attempted to compare the rate of inorganic phosphate uptake and total amount of fixed phosphorous (phosphorous not removed by washing) with the wild type ($C^{+}dg^{S}$) and the resistant variant ($C^{+}dg^{R-1}$), during growth in a mineral glucose medium. Preliminary results indicate that $C^{+}dg^{R-1}$ has less fixed phosphorous per cell during growth in this medium. This would be expected on the basis of the higher activity of the acid phosphatase of $C^{+}dg^{R-1}$ on hexose phosphate esters and the leaching out of the cells of the inorganic phosphate.

2. We have succeeded in producing sufficient rhamnulose to determine whether or not the rhamnose resistant mutant of Salmonella typhosa has lost the ability to produce both rhamnose isomerase and rhamnulokinase, or just fails to produce the latter enzyme because of the absence of the inducing substrate rhamnulose. Several experiments employing rhamnulose as substrate have failed to elicit the production of the kinase. We are therefore left with the alternative that mutation to rhamnose resistance actually results in the genetic inability to produce both isomerase and kinase.

3. Genetic and biochemical experiments with the arabinose negative mutants of Escherichia coli have demonstrated that all mutants isolated can be placed in three closely linked groups (A,B, and C). Recent quantitative enzymatic analysis of extracts of each of the mutants has revealed the following. Each mutant in Group A is deficient in L-arabinose isomerase, an enzyme which catalyzes the conversion of L-arabinose to L-ribulose. All Group B mutants are deficient in L-ribulokinase which catalyzes the phosphorylation of L-ribulose to ribulosephosphate. This group is mixed, however, in that different kinase negative mutants have different L-arabinose isomerase activities, varying from 1/10 to 4 times the activity level of the prototroph. There is no obvious relationship between these differences in isomerase activity in the B locus and the order of the various mutants involved. All mutants in Group C are deficient in both kinase and isomerase.

By a modification of the original procedure, 60 additional arabinose negative mutants have been isolated. Among these are mutants which differ from the former ones studied, in that they are strongly inhibited by arabinose, and probably represent another functional group. The ordering by reciprocal crosses of some of the newly isolated mutants which fall into Groups A,B, and C have presented some difficulty, due to equilibration.

PLANS FOR FUTURE

(a) To determine the nature of the increased phosphatase activity in extracts of Salmonella typhimurium C⁺dg^{r-1}, (b) to determine the relationship between increased phosphatase activity and diauxie, (c) to determine the nature of the metabolic break responsible for the inability of C⁻ mutants to utilize carbohydrates, glycerol, or pyruvate as sole carbon sources, (d) to isolate, map, and characterize enzymatically additional arabinose negative mutants of E. coli, (e) to determine the nature of the effect of a single gene mutation on the activity of both L-arabinose isomerase and ribulokinase.

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EXPERIMENTAL ENTERIC INFECTIONS OF LABORATORY ANIMALS

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ASSISTED BY James Messer, M.S. and K.S. Thind, Ph.D., D.V.M.

TASK NO. NR 103-430

CONTRACT Nonr-2504 (01)

OBJECTIVES

To study experimental enteric infections-
their pathogenesis and protective immunity- in laboratory animals.

ABSTRACT

As mentioned in earlier reports, a continuous flow culture satisfactorily reproduced the in vivo antagonistic relations, as demonstrated in the mouse intestine, between Shigella flexneri and other bacteria. It was found during the current reporting period that continuously filtered effluent from a continuous flow culture of E. coli would inhibit Shigella only when filtration and subsequent growth of Shigella took place in an atmosphere of nitrogen, but not when air was admitted at any time. However, filtrates from continuous E. coli cultures which had been collected in the presence of air regained their inhibitory properties when the redox potential was later reduced by the addition of thioglycollate in a nitrogen atmosphere. Autoclaving of the filtrate did not diminish its inhibitory properties under these conditions. However, growth of Shigella under nitrogen in untreated broth cultures containing equal or greater amounts of thioglycollate was normal. The inhibitory action of E. coli filtrates could be overcome by the addition of glucose. The redox potential of pure or mixed continuous flow cultures of E. coli was about -100 to -150 millivolts. That of pure Shigella or Vibrio cultures about +100 to zero millivolts. It appears that competition for suitable carbon sources under highly reduced conditions is at least one of the basic mechanisms underlying antagonism between Shigella and normal enteric species in continuous flow culture and - by inference - also in the intestine. Minor differences in degree of antagonism exerted by E. coli, Aerobacter sp. and Proteus sp. were not reflected in the inhibitory properties of the filtrates, suggesting that other mechanisms may be present which modify the effect of carbohydrate competition. Some possible mechanisms of this kind, such as CO₂ tension etc. are presently under investigation.

Further experiments by Mr. Messer concerning the mechanism of Candida overgrowth in the intestinal tract during oral administration of antibiotics have indicated that the increase in susceptibility of mice to Candida caused by streptomycin was not abolished by feeding resistant mutants of Clostridia isolated from mouse feces. This completes the survey of major aerobic and anaerobic bacterial species from the intestinal tract. In a total of 30 experiments, replacement of normal enteric flora by antibiotic resistant strains of any of these microorganisms - either in pure or mixed cultures - did not reverse the effect of antibiotic on the susceptibility of mice to oral infection with Candida.

Experiments with 1 to 10 day old chicks indicated that these animals were equally if not more affected by oral administration of antibiotics than mice. Experiments with newly hatched chicks, treated before any enteric flora had been established, indicated that antibiotic will enhance the susceptibility to oral infection with Candida even in the absence of any possible bacterial antagonism. These results appear to rule out bacterial antagonism as a major factor involved in Candida overgrowth during oral administration of antibiotics. In a limited number of experiments, antibiotic administered for the first time together with the inoculum of Candida appeared to be more effective than the same dose of antibiotic given twice on consecutive days prior to infection with Candida. Also, substances irritating the intestinal tract increased the susceptibility of chicks to oral Candida infection. These latter results suggest that the enhancement by antibiotics of the susceptibility to oral Candida infection may be due to an effect on the host rather than to direct stimulation of the Candida.

Studies carried out with Dr. Thind indicated that coproantibody was excreted in guinea pigs and rabbits immunized with intraperitoneal, intravenous or intragastric inoculations of Vibrio cholerae or Salmonella enteritidis. The antibody response was specific and no cross agglutinations could be demonstrated. Intravenous or intraperitoneal immunization resulted in antibody formation both in the feces and serum. Intragastric immunization resulted only in production of fecal antibody while no antibody could be detected in the serum. Examination of intestinal contents of intragastrically immunized animals showed the presence of agglutinin in the duodenum and lower intestinal tract, i.e. in all sites where enteric infections may occur.

PLANS FOR FUTURE

To continue the above projects, especially to study production of coproantibody in human volunteers.

CURRENT REPORTS AND PUBLICATIONS

Thind, K.S., 1959. Studies of coproantibody. Thesis (Ph.D.), J.M.C.
Freter, R., 1959. Study of in vivo and in vitro antagonism between Shigella flexneri and normal enteric flora, Bact. Proc. 12, 97.

PHYSIOLOGY AND BIOCHEMISTRY OF THE NITRIFYING BACTERIA

W. S. Silver
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ASSISTED BY G. W. Griffith

TASK NO. NR 103-431

CONTRACT Nonr-2561(00)

OBJECTIVES

(a) To obtain cell crops of Nitrobacter sufficient for biochemical studies of nitrite oxidation by both intact cells and extracts, (b) to study the mechanism of nitrite oxidation by cell-free extracts, (c) to compare the effect of nitrite on the respiration of Nitrobacter and common heterotrophic microorganisms, and (d) to study the effect of various metabolic intermediates on the respiration of Nitrobacter.

ABSTRACT

Nitrobacter has been grown in sparged carboys in a simple mineral medium under conditions which give a cell crop of ~700 mg (dry wt) per carboy in 4 days at 25° C. The following interesting observations have been made using washed suspensions of whole cells: (1) When nitrite oxidation was measured manometrically, the pH optimum for the oxidation was 7.2 - 7.6. However, when nitrite disappearance was determined chemically, the optimum pH was ca. 5.6. The two methods did not measure comparable phenomena for in the latter uptake rather than oxidation per se was the more rapid process; (2) Cyanide and azide but not 2,4-dinitrophenol or oligomycin inhibited nitrite oxidation; (3) Formate but not acetate, lactate, citrate or glucose was rapidly oxidized. One-half mole of oxygen was taken up for each mole of formate metabolized. The nitrite oxidase of the cell-free system was located in the 'particles' of sonically disrupted cells, but the system was relatively inactive as compared to whole cells.

Many investigators have cited the toxicity of nitrite for microbial growth and respiration, particularly at low pH, for a variety of microorganisms. This was not found to be true for Nitrobacter since cell suspensions completely oxidized nitrite at pH 5.6, albeit at a reduced rate. A comparison was made of the effect of nitrite on the respiration of the following heterotrophic organisms: Escherichia coli, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Hansenula anomala, and Streptococcus faecalis. In all cases nitrite inhibited respiration in the pH range 4.5 - 6.5 but not at pH >7, and inhibition was instantaneous and irreversible. Inhibition could be correlated with the concentration of undissociated HNO₂. It is possible, but unlikely, that the NO formed from HNO₂ decomposing in an acid medium was responsible for the inhibition. However, NO inhibited the respiration of Nitro-

bacter but NO_2^- in an acid medium did not. Spectrophotometric methods have demonstrated that nitrite combined at an acid pH with a cellular enzyme which had a peak of 442 m μ , and that this was not identical with the CO-building enzyme. This evidence supports the working hypothesis that nitrite penetrates microorganisms readily as HNO_2 and immediately combines with a respiratory enzyme, most likely a cytochrome. In Nitrobacter there is no inhibition since it has been shown that the nitrite oxidase is closely linked to the cytochrome system (these Abstracts, 1958).

PLANS FOR FUTURE

(a) To further study the cell-free nitrite oxidase (b) to elucidate the nature of the formate oxidizing system of Nitrobacter and (c) to better define the site of nitrite inhibition of heterotrophic respiration.

CURRENT REPORTS AND PUBLICATIONS

(a) W. S. Silver, "Exogenous respiration in Nitrobacter." Nature, (In the press).

(b) W. S. Silver and G. W. Griffith, "Comparative aspects of the inhibition of microbial respiration by nitrite." Bact. Proc., (Submitted for presentation at Annual Meeting, Society of American Bacteriologists, May, 1960).

(c) W. S. Silver and A. Moscovic, "A review of Soviet research on nitrifying bacteria." Bact. Rev. (In preparation).

THE MICROBIAL CORROSION OF IRON

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ASSISTED BY C. Volkmann, M. Vance, and G. Blanton

TASK NO. NR 103-433

CONTRACT Nonr-375(10)

OBJECTIVES

To study the properties of bacteria which are pertinent to the microbiological corrosion of iron in natural marine environments:

(1) bacterial consumption of oxygen and subsequent production of oxygen differential corrosion cells, (2) the production of corrosive acids, and (3) microbial hydrogenase enzyme activity and its effect on the depolarization of metallic iron surfaces.

ABSTRACT

1. Consumption of oxygen. Seasonal changes in the total organic carbon and oxygen consumption in sediments of the shores and center of Redfish Bay, Texas, a shallow marine bay near the Institute of Marine Science, have been observed. The sediments are made up of distinct layers 10 to 30 mm deep which were sampled to a depth of 20 cm. The organic carbon varied from 0.02 to 4.2 percent in all areas studied. The center of the bays contained more carbon than the edges. The amount of organic carbon in the sediments, available to the bacteria during 40 days of incubation, varied from 0 to 95 percent. The differences in total carbon and available carbon produce distinct layering of oxidized and reduced sediments within a given profile at one station. The sediments are not uniform and it is quite difficult to obtain direct correlation between carbon and seasonal trends. However, in one field area, the surface carbon fluctuated from 1.1 percent in the late fall to 0.3 percent in the early summer. Organic matter accumulates from photosynthetic algae, the processes of sedimentation and the stirring action of the waves on the bottom of the shallow bays. The minimum numbers of bacteria by plate count method was 150,000,000 bacteria per gm in the summer and 1,000,000 bacteria per gm in the winter. No correlation was found between the numbers of bacteria and the available organic matter or loss of organic matter during storage. The differential consumption of oxygen within small depths of the sediment may possibly cause oxygen differential corrosion cells in the presence of iron.

2. Oxygen differential corrosion cells. Preliminary experiments have been conducted on the production of bacterial oxygen differential corrosion cells. A cell has been developed which will permit the study of the production and variations of potentials resulting from bacterial activity. The cell consists of two separate glass chambers between which an iron coupon extends and with the chambers connected with a salt bridge. Calomel and platinum

electrodes extend into the medium of each chamber. One side remains sterile while the other is inoculated with bacteria. The oxygen differential potential can be determined. Tests are in progress.

3. Microcosm experiments. Experiments have been organized to measure the rate of oxygen consumption, the rate of organic matter decomposition, and the production of organic acids in sediments in the laboratory. The experiments are in progress.

4. Corrosion by sulfate-reducing bacteria. Some relationships between bacterial activity and iron corrosion have been determined. Enrichment cultures containing sulfate-reducing bacteria were isolated from sites of active iron corrosion in the marine sediments. Growth studies of the enrichment cultures in peptone-sea water medium were conducted parallel to corrosion experiments, using turbidity as a measure of the number of bacteria. The amount of corrosion was determined by observing the weight loss of iron coupons. Replicate experiments indicated that, although some corrosion existed in sterile medium (0.229 mg/cm^2 in 156 hours), a total of 0.702 mg/cm^2 weight loss in 156 hours was noted in the presence of the bacteria or an increase of approximately 66 percent above controls. The highest corrosion rate was present during the log growth phase.

5. Anaerobic diffusion chamber. A small chamber, consisting of two glass plates separated by a water-tight gasket 1 cm thick containing iron-free semisolid medium, was made to measure the diffusion of iron during anaerobic corrosion. Data show that the iron dissolving from the metal surface diffuses away from the surface. The sulfate-reducing bacteria produce sulfide which combines with the iron to produce a black precipitate which can be observed.

PLANS FOR FUTURE

1. To continue the studies on the consumption of oxygen in sediments and microcosm experiments. 2. To continue tests on corrosion caused by pure cultures of bacteria isolated from corrosion positive enrichment cultures. 3. To continue the study of bacterial oxygen differential corrosion cells. 4. To initiate studies on the effect of hydrogenase on the corrosion of iron.

CURRENT REPORTS AND PUBLICATIONS

(a) C.H. Oppenheimer and L.S. Kornicker (1958) "Effect of the microbial production of hydrogen sulfide and carbon dioxide on the pH of recent sediments." Pub. Inst. Mar. Sci., 5, 5-15.

(b) C.H. Oppenheimer (1959) Bacterial activities in marine sediments. Preprints of papers Gen. Pet. Geochem. Symp., N.Y. p. 49-54.

(c) C. Volkmann (1960) Distribution and decomposition of organic matter in marine sediments. Thesis, University of Texas.

THE MOLECULAR BASIS OF MUTATION

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ASSISTED BY Arthur Dalby, Mary Irving, Gordon Mills, Tadao Horiuchi, Sumiko Horiuchi, Marilyn Thayer, Lei Uemura, Ruth Thielman

TASK NO. NR 103434

CONTRACT Nonr-2771(00)

OBJECTIVES

To understand better the molecular basis of mutation by (a) studying mutations which lead to the production of abnormally large quantities of enzymes, (b) by studying the effect of phosphorous starvation on the genetics and physiology of bacteria, and (c) by the effect of a number of specific compounds and nutritional states on mutation rates.

ABSTRACT

Studies are under way on a mutant of the K12 strain of *E. coli* bacteria. This mutant produces extraordinarily large quantities of a single protein, the enzyme β -galactosidase. Normally, at most, this enzyme constitutes about 4% of the total bacterial protein. In the mutant in question, about 20% of the protein is β -galactosidase.

This mutant strain was obtained by selection in a galactose-limited chemostat operated for many months. At first selection produced a constitutive strain, one which makes β -galactosidase in the absence of the normally needed inducer (lactose, or any one of a number of galactosides). Continued selection resulted in the appearance of bacterial strains having increased enzyme levels. A maximum level now seems to have been reached at about 4 or 5 times the normal value.

We are trying to understand how this bacterium can make more enzyme than the normal bacterium. Is it because it has more copies of the gene required of this enzyme than it does of other genes, or has a mutation occurred which causes the gene to produce more enzyme, either through the formation of more enzyme forming templates, or through the formation of a more active template? Experiments are being carried out to answer these questions and no result is available yet.

Studies are being carried out on the effect of phosphorous starvation on the genetics and physiology of bacteria. During phosphorous starvation there is a breakdown of RNA with an attendant synthesis of DNA. The effect of this starvation on mutation rates is being studied. For the present, most attention is being paid, however, to the study of the effect of phosphorous starvation on the induced synthesis of certain enzymes. We have found that the synthesis

of some enzymes can be induced during the phosphorous starvation phase. We would like to conclude from this and other observations that the phenomenon of enzyme induction, i.e., the regulation of the rate of protein synthesis occurs at the level of formation of enzyme by template rather than at the level of formation of templates by the gene.

A second problem being studied here is the physical relationship between RNA and DNA within the bacterium. During phosphorous starvation, RNA falls until it reaches a value where the ratio of RNA to DNA is 1. We are looking to see whether this results from a specific physical association between RNA and DNA under these conditions. This is being done by the use of certain nucleic acid specific fluorescent stains. Studies are under way on the effect of certain newly discovered antibiotics on mutagenesis in bacteria. These include mitomycin C, which specifically blocks DNA synthesis and psicofuranine, which is an analogue of adenosine.

THE NUTRITIONAL REQUIREMENTS FOR SPOULATION
IN THE BACILLUS GENUS AT A CELLULAR LEVEL

Roy M. Johnson
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ASSISTED BY (part-time) Carl E. Cords

TASK NO. NR 103-435

CONTRACT Nonr-2794(01)

OBJECTIVES

(a) To establish amino acid or inorganic nitrogen requirements for sporulation in the genus Bacillus at the cellular level, (b) to study the sequence from vegetative cell to spore under varied nutritional conditions as regards nitrogen metabolism.

ABSTRACT

Nine strains of Bacillus cereus and twelve other species of this genus have been shown to grow and sporulate on a synthetic medium containing five amino acids, glucose and EDTA. Vitamins were not required by any of these species.

Since one of the indirect objectives of this study was to demonstrate the relationship, if any, of sporulation to a sexual process, advantage was taken of the fact that the above medium gave sporulating cells in the pellicle and non-sporulating cells in the body of the medium to test the effect of iodate on the sporulation process. In serial dilution, iodate retarded pellicle formation and was subsequently shown to quantitatively inhibit sporulation for two species. This was related to the established ability of iodate to oxidize receptors in other biological systems and taken as indirect evidence of the sexual nature of bacterial spore formation.

Microscopically, living cells, simple stained cells and nuclear-stained cells grown on nutrient agar, the above synthetic medium and in the presence of iodate, have been followed photographically. Nuclear elements appear swollen in the presence of iodate, and the relation of this to sporulation is under investigation. Single cell cultures have not, as yet, been successfully grown under continuous microscopic observation. Lucite rods are being tried to increase the light intensity while reducing the heat effect of microscopic illumination.

PLANS FOR FUTURE

(a) To continue technique improvement in culture of individual cells under phase microscopy (b) to correlate this with nuclear changes (c) to follow amino acid metabolism by paper chromatography in the synthetic medium described.

CURRENT REPORTS AND PUBLICATIONS

- (a) C. E. Cords and R. M. Johnson (1959), "Sporulation studies. I. A synthetic medium for sporulation in the genus Bacillus." Submitted J. Bacteriol.
- (b) R. M. Johnson (1959), "Sporulation studies. II. The effect of KIO_4 on sporulation in the Bacillus genus." Submitted J. Bacteriol.

HYPERSENSITIVE REACTIONS OF LABORATORY ANIMALS TO
ASCARIS LUMBRICOIDES

K.A. Laurence
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ASSISTED BY

TASK NO. NR 103-440

CONTRACT Nonr-2725(00)

OBJECTIVES

a) To develop methods to stimulate in vitro hatching of A. lumbricoides larvae, b) to axenically cultivate the hatched organisms as source material for studies on (1) physiological and morphological development of the larvae, and (2) effects of antisera, drugs, and antimetabolites on the developing worm.

ABSTRACT

Past experiences have shown that animals actively infected with *Ascaris* larvae demonstrate both an immediate and a delayed type skin reaction upon testing with the larval antigen. All immunized animals however, respond only with the immediate skin reactions with the egg, larval or adult perienteric fluid testing agents. Attempts to separate and determine the components of the soluble testing agents responsible for these skin reactions were made by electrochromatographic separation procedures and biochemical identification of each fraction. Twenty-two separated fractions were tested for the presence of skin reactive substances for the presence of carbohydrate and for the presence of protein. Two electrophoretically distinct fractions were found to contain the skin reactive substances which elicit the immediate skin response in infected or immunized animals. The larval extract which elicits the delayed type skin reaction in infected animals only, is not as clearly separated.

In addition, the skin testing agents, prepared by sonic oscillation of viable eggs or larvae, contain substances which can, with proper concentration, cause retardation of development, agglutination, and finally lysis of normal, but unembryonated *Ascaris* eggs. Further analyses of the chemical and physical properties of these agents are now in progress in an attempt to concentrate and identify these substances.

Methods have been developed to stimulate in vitro hatching and axenic cultivation of the hatched larvae. The cultivated larvae can be maintained in a chemically defined medium for nearly two months with little mortality. Even though the larvae remain viable, little change in overall size or internal cellular structure has yet been observed.

Antiserum, prepared in rabbits against larvae will immobilize and kill the axenic grown larvae in the presence of guinea pig complement. The mechanism of action of the antiserum has not yet been determined.

PLANS FOR FUTURE

(a) To expand the investigation of the in vitro cultivation of hatched *Ascaris* larvae (b) to determine the site of reaction of antilarval sera on the larvae by fluorescently labelled antibody (c) to study the morphological development of the larvae by electronmicroscopy.

CURRENT REPORTS AND PUBLICATIONS

(a) K.A. Laurence "Hypersensitive Reactions of Laboratory Animals to Ascaris lumbricoides var. suum Fed. Proc. 18: (Part 1) 579 (1959).

RESISTANCE TO DISEASE: METHODS OF MEASUREMENT AND
EXPLORATION OF DRUGS TO INCREASE RESISTANCE

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ASSISTED BY J.L. Cutler, M. Kojima, and V.Z. Pasternak

TASK NO. NR 103-448

CONTRACT Nonr-2610(01)

OBJECTIVES

(a) The isolation and identification of microbial lipids which stimulate the reticuloendothelial system (RES) to hyperfunction, (b) the development of high immune states or the increase of normal host defense, and (c) expansion of quantitative testing methods for the RES.

ABSTRACT

The fractionation of lipids from yeast has continued; and although this still represents a mixture, considerable progress has been made in reducing the number of different lipids. The efficacy of this material to increase antibody titer has increased well above the five times reported previously. For instance, in botulinum toxin challenge, the animals given the lipid plus toxoid can withstand one hundred times the dose of control animals which received toxoid alone. Histological studies have indicated that the first obvious response to the lipid is a hyperplasia of alveolar septal cells and perivascular cells. These RE cells and the very characteristic and unusual picture they present is identical to that seen when the crude cell wall fraction of yeast is given. It appears that these lung elements may play a very significant role in increasing host resistance. All attempts at successfully tagging polystyrene spheres with an isotope other than tritium have been unsuccessful due to the difficulty of resuspending polystyrene spheres after precipitation. Consequently, we are now in the process of trying to nitrate the benzene ring and follow this by diazotization in hopes of putting labeled protein on to the surface of the sphere.

PLANS FOR FUTURE

(a) Continuation of the fractionation in order to attempt to isolate and identify the specific lipid involved, (b) determine whether or not the stimulation of the RES is due to a humoral factor whose release is triggered by the lipid, and (c) continuation of the work with polystyrene spheres.

CURRENT REPORTS AND PUBLICATIONS

The following papers are in press:

(a) J.H. Heller, "The effect of zymosan on the RES." Proc.

Third International Sym. on RES, Ronald Press, New York.

(b) J.L. Cutler, "The enhancement of hemolysin production in the rat by zymosan." J. Immunol.

(c) J.H. Heller, "Nontoxic RES stimulatory lipids." Ann. N.Y. Acad. Sci.

(d) M. Kojima, "Morphologic changes accompanying RES stimulation." Ann. N.Y. Acad. Sci.

TEST OF A NEW HYPOTHESIS FOR THE ETIOLOGY OF
RHEUMATIC FEVER

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ASSISTED BY P. N. Lowry

TASK NO. NR 103-454

CONTRACT Nonr-2837(00)

OBJECTIVES

This research is designed to test the possible application of the clonal selection theory of antibody production of Burnet and Fenner as an explanation of the etiology of rheumatic fever.

ABSTRACT

Briefly, the hypothesis as it might apply to rheumatic fever implies that the preceding streptococcal infection creates an environment in the pharyngeal lymphoid tissue which allows the production of clones of cells which produce a "new antigen" possibly incorporating connective tissue components as a hapten. This new antigen would circulate and be deposited at distant sites. Antibody would be formed against this "new antigen" and antigen-antibody reactions would then occur in connective tissue sites such as joint linings, endo-, myo-, and pericardium. This hypothesis fits the time scale and natural history of rheumatic fever. It is proposed to test this hypothesis in the following way. Tonsils from a child with acute rheumatic fever would be expected to contain the "new antigen." A skin test material prepared from the lymphoid cells of the tonsillar tissue and injected intradermally into subjects who have or have had rheumatic fever and a control group of non-rheumatic subjects, might result in different reactions in the two groups.

Lymphocytes were obtained from tonsillar tissue of a child with acute rheumatic fever 5 days after the onset, and from another non-rheumatic child. Skin test antigen containing 1:100 and 1:1000 lymphocytes by volume in sterile normal saline were prepared. These preparations were rendered bacteriologically sterile by incubating for two hours with penicillin, streptomycin and mycostatin. The results are shown in tabular form below. Obviously it is too early to say much of the results so far. More testing will be needed and it is possible that living lymphocytes rather than the remains of frozen lymphocytes should be used as the skin-test antigen. It is also possible that the connective tissue cells of the tonsils would be a better antigen.

	Rheumatic Antigen		Non-Rheumatic Antigen	
	Pos.	Neg.	Pos.	Neg.
Rheumatic Subjects	1	19	1	14
Non-Rheumatic Subjects	4	13	3	14

PLANS FOR FUTURE

(a) To obtain more tonsils from patients with acute rheumatic fever, (b) to prepare two antigens, one with living lymphocytes and the other with connective tissue cells, (c) to skin test a group of rheumatic and non-rheumatic subjects with these new antigens, (d) to continue skin testing rheumatic and non-rheumatic subjects with the present antigen.

AN INVESTIGATION OF SOME INTERACTIVE PHENOMENA
AMONG MICROORGANISMS

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ASSISTED BY E. C. S. Chan, N. R. Krieg, D. A. Power

TASK NO. NR 103-455

CONTRACT Nonr-595(12)

OBJECTIVES

To discover physiological phenomena (other than anti-biosis) that are peculiar to mixed microbial populations, following which they will be analyzed in an attempt to obtain an explanation for the observed effects. Currently, phenomena associated with sporulation and nutritional symbiosis are being studied.

ABSTRACT

(a) In the symbiotic system involving Lactobacillus plantarum and Streptococcus faecalis, attention was directed to the biosynthesis of the pteroylglutamic acid-like compound synthesized by the lactobacillus. p-aminobenzoic acid was a requirement for this synthesis. Pteroylglutamic acid has been postulated either to be this synthesized compound or an intermediate in its production. Therefore it was desirable to determine if pteroylglutamic acid could replace the p-aminobenzoic acid requirement of the lactobacillus. This question has been controversial in the literature. In the present work, the inhibition of the growth of the lactobacillus by high concentrations of sulfathiazole was reversed non-competitively by pteroylglutamic acid in a chemically-defined broth medium, but only after prolonged incubation. When a cell suspension was plated in an agar medium of similar composition, containing pteroylglutamic acid and sulfathiazole, daily counts from the same plate revealed increasing numbers of colonies up to the seventh day, when the count approached the total number of cells plated. This total recovery of the organisms indicated that a mutational event was not involved in the delayed reversal. The reversal was not due to degradation of the pteroylglutamic acid to p-aminobenzoic acid, p-aminobenzoylglutamic acid, or pterioic acid, nor could it be attributed to a typical adaptive process. The delay could be explained by recent work in the field which indicates that L. plantarum lacks the enzyme system necessary to reduce pteroylglutamic acid to active forms.

(b) Cell-free filtrates of Erwinia atrosepica, obtained from a chemically-defined medium, were characterized with reference to their capacity to enhance sporulation of Bacillus sphaericus. The active principle(s) could be

removed by charcoal adsorption but it could not be recovered by elution. Of 24 chromatographic materials tested, only a strong anion exchange resin would remove the activity. In an analysis for fermentation end-products the activity could not be detected in either the residue or the distillate on initial alkaline distillation. Concentration of the factor(s) was not possible by freezing followed by thawing. Although an absorption peak at 276 m μ was obtained in some batches of filtrate, this was found to be unrelated to the sporulation activity. The activity was destroyed by boiling for 30 minutes. It decreased on autoclaving and was destroyed by acid and alkaline hydrolysis. Dialysis removed all activity. The activity could be concentrated by vacuum distillation. Paper chromatography and bioautography of the active residue from vacuum distillation suggest that the phenomenon may involve more than one chemical entity.

(c) An attempt to demonstrate the growth of obligate thermophilic bacteria under mesophilic conditions in association with mesophilic bacteria is being made. Six obligate thermophiles used as indicators have been incubated at 37 C with mesophiles isolated from different types of soil. Techniques thus far employed have included the use of a semi-permeable membrane to separate the two types of organisms while allowing diffusion of any active principles, and also the use of extracts of mesophilic organisms in penicylinder experiments. Thus far none of the indicator organisms have responded in the experimental systems.

PLANS FOR FUTURE

(a) Chromatographic and bioautographic techniques will be employed in the characterization of the folic acid-like factor produced by L. plantarum. A study of the fermentation end-products of the lactobacillus and streptococcus, individually and in symbiotic growth, will be made.

(b) Continued effort will be directed toward the isolation and identification of the sporulation enhancement factors produced by Erwinia atroseptica.

(c) Further techniques will be employed in the investigation of the effect of microbial association in thermophily.

THE GENETICS AND RADIOBIOLOGY OF PARTIALLY
RESPIRATION DEFICIENT SACCHAROMYCES

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University of Wichita

ASSISTED BY G. Fowler and R. Rule

TASK NO. NR 103-459

CONTRACT Nonr-201(02)

OBJECTIVES

(a) To obtain further information bearing upon the question of whether nuclear or cytoplasmic damage is fundamentally responsible for the respiratory deficiency induced by ultraviolet radiation in Saccharomyces and (b) to examine various cultural conditions influencing the differentiation for respiratory capability which occurs within yeast populations.

ABSTRACT

(a) Analyses have been completed of the induction by ultraviolet light and of the photoreactivation of respiratory deficiency in a polyploid family of Saccharomyces. The results established that the number of radiation events required to induce defective respiration as well as the extent of inducing damage which is photoreparable can be related integrally to cellular DNA contents and levels of ploidy; no correlations with cellular RNA contents were uncovered. These findings reinforce the hypothesis (Cytologia 23:143, 1958) that ultraviolet induced loss of respiratory ability in yeasts is instigated by nuclear disturbances.

(b) A unique group of ultraviolet induced partially respiration deficient yeasts have been obtained. These mutants may undergo differentiation to either complete respiratory sufficiency or deficiency whenever mitotic, caryogametic or meiotic activity occurs. Genetic analyses indicate that the mutant character is chromosomally linked and probably originates through a heterochromatic defect.

(c) We have reported that Mn^{++} induces respiratory deficiency in non-dividing yeast cells through some derangement of their utilization of amino acids (Biochim. et Biophys. Acta 33:227, 1959). Examinations of the effects of this ion upon actively growing cultures established the following: (1) Over a range of Mn^{++} concentrations, inhibition of growth and induction of respiratory mutants occurs only when the molar ratio of Mg^{++} to Mn^{++} is 1:4 or less. (2) Respiration deficient mutants are relatively Mn^{++} resistant; at inducing Mg^{++}/Mn^{++} concentrations a positive growth selection for mutants exists. (3) Mn^{++} , Fe^{++} or Fe^{+++} can alleviate both growth inhibition and mutation caused by Mn^{++} . Ca^{++} has no effect. The fact that the reversing ions are essential for cytochrome syntheses suggests that Mn^{++} , like other inducers of respiratory deficiency, exerts its mutagenicity by interfering with the cells ability to form the terminal respiratory chain. (4) Induction and selection occurs for a group of partially respiration deficient cells. Such mutants are more resistant than totally deficient cells to growth inhibition by Mn^{++} and are refractory to transformation into totally deficient cells in the presence of Mg^{++}/Mn^{++} concentrations

which have a pronounced inducing effect upon normal individuals. In contrast to the intermediate respiratory types produced by ultraviolet, these induced by Mn^{++} are completely stable during vegetative growth.

(d) Respiratory deficiency is induced when yeast cells unadapted to aerobic respiration are grown in the presence of the cysteine antagonist allyl glycine. Respiration adapted cells do not mutate. Cysteine specifically reverses allyl glycine mutagenesis while homoserine enhances mutation. Homocysteine, methionine and alpha-amino-N-butyric acid also reverse mutation, but to a lesser extent than cysteine. The comparative effectiveness of these amino acids corresponds to their relative placement in the biosynthetic sequence leading to cysteine. The over-all findings indicate the existence of a physiologic function centering about cysteine metabolism which is crucial for the genetic maintenance of the aerobic respiratory apparatus.

PLANS FOR FUTURE

(a) to study ultraviolet induction of partial respiratory deficiency in polyploid Saccharomyces, (b) to compare ultraviolet induction of total respiratory deficiency in respiration competent and partially deficient cells, (c) to evaluate the interaction of Mn^{++} and allyl glycine in induction of respiratory deficiency and (d) to investigate the influence of cultural conditions upon the differentiation for respiratory capacity occurring among ultraviolet induced partially respiration deficient mutants.

CURRENT REPORTS AND PUBLICATIONS

(a) Sarachek, A. (1959), "Induction by ultraviolet radiation of heritable respiratory deficiency in polyploid Saccharomyces". Annual Meetings, M.V. Branch, Soc. Am. Bact., Lincoln, Nebr., April 19, 1959.

(b) Sarachek, A. (1959), "The induction by Mn^{++} of heritable respiratory deficiency in non-dividing populations of Saccharomyces". Biochim. et Biophys. Acta, 33:227-230.

(c) Sarachek, A., "The induction by ultraviolet radiation and the photoreactivation of heritable respiratory deficiency in polyploid Saccharomyces". Cytologia, in press.

(d) Sarachek, A., "The induction and selection by Mn^{++} of respiration deficient Saccharomyces". Microbial Gen. Bull., in press.

(e) Sarachek, A., and Fowler, G.L., "The induction by allyl glycine of heritable respiratory deficiency in Saccharomyces and its reversal by sulfur amino acids". Can. Jour. Microbiol., in press.

NUCLEIC ACID METABOLISM IN NEUROSPORA CRASSA

Val W. Woodward
University of Wichita

ASSISTED BY Wilda Cook

TASK NO. NR 103-461

CONTRACT Nonr-2772(00)¹

OBJECTIVES

(a) To elucidate the genetic control of pyrimidine metabolism in Neurospora crassa, and (b) to study the nature of one of the genes involved as regards its fine structure, i.e., its ultimate mutational, re-combinational, and functional sub units.

ABSTRACT

Classical genetic theory tells us that mutant x mutant crosses involving a x a, or b x b, etc., give rise only to a, b, etc., progeny. The theory is based on analyses of pedigrees with relatively small numbers of offspring, and only rarely has the investigator found unexpected types among the progeny of a given cross; usually the exceptions have been discarded. In recent years, however, with the rapid growth of microbial genetics, the occurrence of unexpected types is common enough to provoke a reevaluation of classical theory.

The present study pertains to the analysis of the Pyr-3 locus of Neurospora crassa. The Pyr-3 locus is one of five loci governing the synthesis of uridine nucleotide (the details of the genetic control of pyrimidine metabolism have been reported earlier). More than 100 mutants of the same apparant phenotype, and allelic by standards of classical genetics, have been isolated. To date, 32 of these have been studied genetically.

On crossing the 32 "allelic" mutants in all possible combinations with one another, it was found that certain combinations yielded, in addition to the expected mutant progeny, a small number of prototrophs. Again, relying on genetic tradition, one predicts either that mutation or crossover is the mechanism giving rise to these prototrophs. Since mutation was ruled out (on the basis of frequency), the other alternative was investigated. On the premise that crossover is responsible for the prototrophs, its becomes readily apparant that the Pyr-3 region, like the whole chromosome, possesses the property of linearity. A cross between two mutants with the same gross phenotype is capable of giving rise to prototroph progeny if, and only if, the mutant lesion in one does not overlap, along a one dimensional plane, with the lesion in the other, permitting chromatid exchange between the two lesions.

The 32 mutants investigated indeed bear out the above prediction. At least 13 separable sites within the Pyr-3 region have been mapped, the entire region exhibiting the same sort of linearity we have come to associate with whole chromosomes. Within the group, certain of the mutants do not give rise to prototrophs when crossed with other mutants; these have

been interpreted as being allelic. Other mutants appear to be characterized by lesions extending over a distance of two to six sites. But, the significant point is that additivity maintains throughout the entire region.

At the present time analyses are being made of the functional properties of discrete sections of the Pyr-3 region. Functional homology is determined by the heterocaryon method; i.e., if two mutants within one heterocaryon maintain growth in the absence of a pyrimidine supplement, they represent mutations of distinct functional parts, but if they do not maintain growth then the same functional lesion is involved. The 32 mutants, in all possible heterocaryon combinations, show the existence of at least 5 functional groups within the Pyr-3 region. Since a negative heterocaryon test is not conclusive, it is premature to state that the ultimate functional groups of the Pyr-3 region have been "mapped", but, to date, there is a perfect correspondence between the linearity of the mutational sites and the functional groups.

PLANS FOR FUTURE

(a) To continue the study of mutational and functional sites within the Pyr-3 region, and (b) to apply the same techniques toward the study of another locus (one that has shown on preliminary examination to be slightly different from Pyr-3).

CURRENT REPORTS AND PUBLICATIONS

(a) Suyama, Y., Munkres, K.D., and Woodward, V.W. (1959), "Genetic analyses of the Pyr-3 locus of Neurospora crassa: the bearing of gene conversion and recombination upon intraallelic linearity." Genetica, November, 1959 (in press).

(b) Suyama, Y. and Woodward, V.W. (1960), "Functional sites within the Pyr-3 locus of Neurospora crassa." In preparation.

(c) Woodward, V.W. (1960), Abstract of functional sites within the Pyr-3 locus of Neurospora crassa. To be presented to the AIBS, August, 1960.

BACTERIAL AND VIRAL GENETICS

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ASSISTED BY N. Yamamoto, D.H. Walker, Jr. and R. Stephens

TASK NO. NR 103-462

CONTRACT Nonr-2791(00)

OBJECTIVES

(a) To elucidate the mechanisms of synthesis of bacterial viruses, and (b) to study the relations between the genetics of viruses and of their host cells.

ABSTRACT

A method for making very thin carbon membranes for supporting electron microscope specimens has been developed. It consists of evaporating carbon on a freshly-cleaned mica surface and dipping the mica into a formvar solution. The formvar and carbon is then stripped from the mica onto a water surface and picked up on electron microscope grids. The formvar is then dissolved in ethylene dichloride leaving the carbon membrane on the grid.

The morphology of T2 bacteriophage as seen by Brenner and Horne (The Times (London) Science Review, Autumn 1959, p. 10) has been confirmed using their "negative staining" technique. In this technique, the specimen is allowed to dry on the grid in the presence of an excess of electron dense phosphotungstic acid which dries around the specimen. The increased contrast thus gained permits one to distinguish many new details in T2 phage particles: The head is connected to a hollow "spike" (1000 by 70A) which is surrounded by a sheath, (160 A in diameter) and terminated by an adsorption "plate" to which long fine (30A) fibers are attached. When the particle encounters a host cell the sheath contracts to form a helically grooved cylinder about 370A long and 300A in diameter. This exposes about 570A of the spike which presumably travels through the bacterial cell wall and serves as a needle through which passes the DNA which had been packed in the phage head.

Using this technique we have begun an examination of T5 bacteriophage. Its construction seems much simpler than that of T2, consisting of a head about 900A in diameter to which a 1800A long, 100A wide tail is attached. The tail is hollow with a 30A bore and under some conditions seems to be quite flexible.

PLANS FOR FUTURE

(a) To examine the detailed morphology of other bacteriophages in the "T" set, and (b) to determine how their structural elements function during infection of host bacteria.

CURRENT REPORTS AND PUBLICATIONS

(a) T. F. Anderson (1959), "Bacterial Viruses - Structure and Function." in Stanier and Gunsalus, Eds. THE BACTERIA, Academic Press, New York, Vol. 1, pp. 387-414.

(b) A. Lwoff, T. F. Anderson and F. Jacob (1959), "Remarques sur les caracteristiques de la particule virale infectieuse." Ann. Inst. Pasteur, 97:281-289.

AN INVESTIGATION OF SOME HALOPHILIC BACTERIA

Dr. H. O. Halvorson, Responsible Investigator

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Dr. K. G. Gollakota

ASSISTED BY John DePinto & Leena Narasimhan

TASK NO. NR 103-463

CONTRACT NONR-1834(30)

OBJECTIVES

Development of Media, Technique for Counting, Studies of the salt concentration within and outside of the cells, Studies of white halophile, Attempts to obtain mutants.

ABSTRACT

Media have been developed that permit rapid growth of *Pseudomonas salararius*. The best medium is one made up with 0.5% trypticase, some yeast extract, 2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 25% cp NaCl. Good growth has also been obtained in a chemically defined medium containing 21 different amino acids. Of these arginine, valine, isoleucine and lysine are essential and the other 21 are stimulatory.

Pseudomonas salanirus will not grow unless the salt concentration is above 17%. An unknown pigmentless isolate which was formerly thought to be a mutant of the former will grow well in media where the salt concentration varies from 1% to 23%. Attempts to repeat the finding of this so called mutant have not been successful. We have also been unable to develop mutants with ultra-violet light treatment.

An analysis of the salt concentration in the medium and within the cells of *Ps. salanarius* have shown that the concentration of NaCl within the cell is essentially the same as in the outside medium.

STUDIES ON THE NATURE OF IMMUNITY
AND PARASITE SPECIFICITY IN HELMINTHS

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ASSISTED BY G. Hsu, G. Harrington, D. Doro, A. Williams

TASK NO. NR 103-466

CONTRACT Nonr-233(56)

OBJECTIVES

(a) To study the immune process in the tapeworm Hymenolepis nana in metabolic terms, (b) to develop respirometric techniques suitable for tapeworm cysticercoids using a modified Cartesian Diver, (c) to study embryological processes in tapeworms under varying hostal conditions, (d) to undertake a comparative study of helminths in terms of variations in host and parasite distribution.

ABSTRACT

Primary emphasis since January 1, 1959 on the research program supported by this contract has been the development and use of a highly sensitive microrespirometer of the Cartesian Diver type specifically adapted to parasitological research material. Since no comparable studies have been undertaken, it has been necessary to develop a new regimen of appropriate techniques. Shape and size of the diver, volume standardization, and loading procedures have all been considerably modified and finally standardized to the point where systematic comparative data are becoming available. A total series of twenty experimental runs have been undertaken, each utilizing a set of eight divers containing cysticercoids of Hymenolepis nana in various experimental media. The tests to date have attempted to establish a standard metabolic gradient for the mature larvae, utilizing both a simple glucose medium for general activity rates at a given temperature over a range from 8-36 hours, and a specific substrate for a particular enzyme system (succinic dehydrogenase).

One of the more interesting observations to date is that glucose does not appear to stimulate total oxygen uptake over the first 24-hour period. There appears to be sufficient endogenous glycogen stored in the tail and membranes as shown in other studies (cf. Heyneman and Voge, Journ. Parasit. 43: 527-531, 1957) to maintain the normal rate of activity for the full 24-hour period. By the end of this period much of the tail glycogen is consumed. It is noteworthy that infectivity in the final host stops at about 24 hours, despite the fact that metabolic activity continues as long as 48 hours. ATP and glucose-phosphate dependence have also been studied. Bactericidal agents are not needed during these relatively short-term experiments.

Correlated with these studies are other investigations of host specificity in helminths. A large series of helminth whole mounts have been made for a study of the distribution and specificity of these organisms in their natural hosts. Work was completed and manuscripts submitted on tetrazolium enzyme studies in H. nana and antoreinfection in H. nana. A technique of gross tapeworm dissection was also developed.

A mutant form of Hymenolepis nana has been discovered and various experiments with this anomaly undertaken. In addition, an analysis of evolutionary implications from a study of host-specificity in the nematode genus Cyrtosomum has been completed and the manuscript submitted; a theoretical account of evolution among the digenetic trematodes was submitted for a special commemorative volume.

PLANS FOR FUTURE

(a) Present plans call for a curtailment of the current research direction in order to participate in a larger-scale study on immunological aspects of disease in the Sudan (Task NM 52 11 03.6 - Field Studies on Kala Azar and other Diseases in the Sudan). It is anticipated that these studies on immunological, nutritive, public health and other aspects of Kala Azar will require a three-year program (b) continuation of H. nana Cartesian Diver respirometric studies by Mr. G. Harrington, graduate student, with emphasis on temperature-controlled metabolic rates in tapeworm larvae.

CURRENT REPORTS AND PUBLICATIONS

(a) D. Heyneman (1959), "Experimental autoreinfection of Hymenolepis nana in isolated mice restrained from coprophagy." (abstract) J. Parasit. 45, 4 sec. 2, 25.

(b) D. Heyneman (1959), "A population of branched tapeworms (Hymenolepis nana) found in a strain of DBA-1 mice." (abstract) J. Parasit. 45, 4 sec. 2, 25.

(c) D. Heyneman and M. Voge (in press), "Succinic dehydrogenase activity in cysticercoids of Hymenolepis measured by the tetrazolium technique." Exper. Parasit.

(d) D. Heyneman (in press), "Cuticular peeling - a dissection technique for preparation of cestode whole mounts." J. Parasit.

(e) D. Heyneman (in press), "On the origin of complex life cycles in the digenetic flukes." (festschrift volume for Prof. Eduardo Caballero y C.).

(f) J. J. Gambino and D. Heyneman (in press), "Specificity and speciation in the genus Cyrtosomum (Nematoda: Atractidae)." Amer. Midl. Nat.

(g) M. Voge and D. Heyneman (MS submitted), "Studies in cysticercoid histology II. Observations on the fully developed cysticercoid of Hymenolepis nana (Cestoda: Cyclophyllidae)." Submitted Nov. 20, 1959 to Proceedings Helminthological Society.

IMMUNOLOGICAL INVESTIGATIONS ON THE PITUITARY
GLAND OF THE RAT

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ASSISTED BY Dorothy M. Anigstein

TASK NO. NR 103-981

CONTRACT Nonr-2564(00)

OBJECTIVES

To study the immunological properties of the antisera against the anterior lobe of the pituitary.

ABSTRACT

Previous observations of the authors on the biological properties of the antirat pituitary serum (APS) injected into rats indicated a relative suppression of body-weight (Anigstein, Whitney and Rennels, 1958).

The purpose of the present investigations was to study the experimental conditions under which the hormones present in the tissue homogenate of the rat pituitary can be influenced by the homologous antiserum. Parallel observations were made on the activities of antibodies elicited by purified bovine growth hormones used as antigens. Consequently, it was possible to compare the immunological relationships of the rat pituitary tissue and APS with the purified growth hormone preparations and their antisera. The experimental assay used in this study gave us the opportunity to investigate not only the effect of the pituitary antiserum (APS) upon the growth stimulation of hypophysectomized rats, but also its antigonadotropic and antithyrotropic activities. Thus an increase of ovarian and uterine weights in animals given pituitary homogenate is the reflection of gonadotropic stimulation, while an increase in thyroid weight similarly reflects thyrotropic activities. Inasmuch as these organs were weighed routinely, it was possible to detect stimulation by the hormones or inhibitory effects by antihormones by comparison with the weights of the organs of control rats. In addition to the above in vivo studies, parallel serological tests in utilizing complement fixation, precipitin ring tests and agar diffusion techniques were utilized.

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Office of Naval Research. Report ACR-47.
PROGRESS REPORT ABSTRACTS - Microbiology
Branch, 139 pp. January 1960.

This publication consists of a collection of 71 short progress reports prepared by the investigators sponsored by the Microbiology Branch of the Office of Naval Research. It is designed to meet a need for reciprocal exchange of conveniently summarized research data among these investigators. Subjects covered may fall under one or more of the following generalized topics.

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(2) Action of antibiotics, antiseptics, germicides, and
chemotherapeutic agents, (3) Marine microbiology,

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